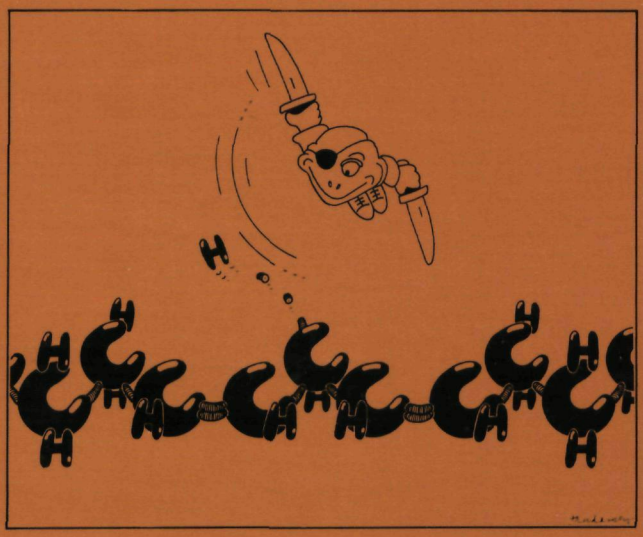
THE AUTOXIDATION OF MODEL MEMBRANES



HUGO WEENEN

THE AUTOXIDATION OF MODEL MEMBRANES

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THE AUTOXIDATION OF MODEL MEMBRANES

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
IN DE WISKUNDE EN NATUURWETENSCHAPPEN
AAN DE KATHOLIEKE UNIVERSITEIT TE NIJMEGEN
OP GEZAG VAN DE RECTOR MAGNIFICUS PROF DR P G A B WIJDEVELD
VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN
IN HET OPENBAAR TE VERDEDIGEN OP DONDERDAG 13 MEI 1982
DES NAMIDDAGS 2 UUR PRECIES

DOOR

HUGO WEENEN

GEBOREN TE TILBURG

krips repro meppel

We shall not cease from exploration
and the end of all our exploring
will be to arrive where we started
and know the place for the first time

T.S. Elliot

For Gerda

For my parents

For family and friends

ACKNOWLEDGMENTS

Scientific research can be a breathtaking adventure, including the sleepless nights spent on endless experiments, the frustrations associated with instrument stubborness, and writers block when trying to make sense out of promising but not totally decisive data. However, solidarity among fellow adventurers has been a learnful experience that goes beyond the intrinsic value of research.

I would like to thank everybody who helped to make this such a worthwhile experience, in particular Voula Kontouyannidou, Carl B. Ziegler, Jamil A. Khan, Bob Mebane, Yogi Wolfram, Farid and Liliane Khouri, Karl Smith, Rick Wolf, Bruce Weber and Rob Schemkes. I would also like to thank Peg Musser for both her skill and patience in helping me prepare this manuscript, and Jan Damen for the artwork on the frontpage.

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Part of the results described in this dissertation have been published or are accepted for publication:

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N. A. Porter, R. A. Wolf, E. M. Yarbro, and H. Weenen

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Chapter 4

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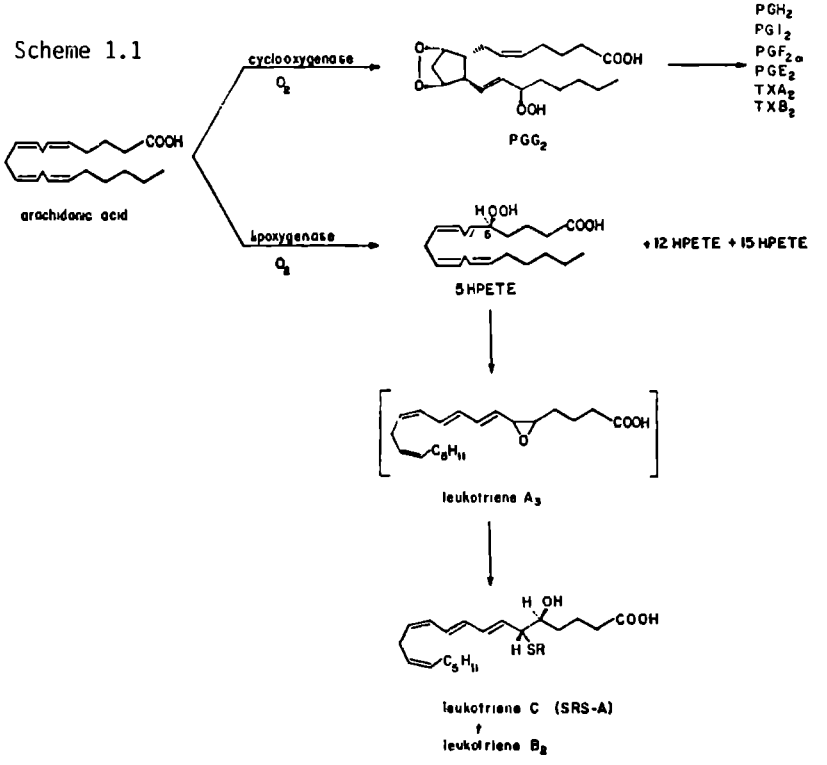
CHAPTER 1

INTRODUCTION

1.1 LIPID OXIDATION

Lipid peroxidation is a biologically important process. The synthesis of prostaglandins (PG's) activated by the enzyme cyclooxygenase and lipoxygenase mediated lipid hydroperoxide formation are important physiological processes. They are essential for blood platelet aggregation, smooth muscle contraction, the inflammatory process and phagocytosis (1-7).

Scheme 1.1



Non-enzymatic oxidation of lipids may also play an important biological role. In fact, a theory of aging has been proposed based on lipid peroxidation. Both the aging of normal erythrocytes as well as the formation of age pigments, it is suggested, are related to the formation of lipid peroxides(8,9). As much as enzymatic lipid oxidation is essential for a normally functioning living species, random oxidation of lipids can pose a great danger when the subtle balance between oxidative and anti-oxidative mechanisms is disturbed. This is clearly demonstrated by the fact that mammals are killed when exposed to oxygen concentrations only five or ten times higher than that present in air. The very oxygen which is essential to our lives also poses a great danger, and we survive only because of elaborate defense mechanisms that inhibit oxidation(10). It is therefore not surprising that many diseases seem to be caused by excessive oxidation, which occurs when the protection mechanisms against oxygen are not properly functioning(11-18).

Scheme 1.2

initiation $LH + In\bullet \rightarrow L\bullet + InH$ (1)

propagation $L\bullet + O_2 \rightarrow LOO\bullet$ (2)

$LOO\bullet + LH \rightarrow LOOH + L\bullet$ (3)

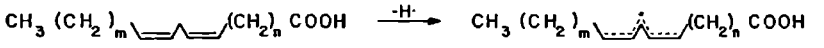
termination $LOO\bullet + LOO\bullet \rightarrow LOOL + O_2$ (4)

The process by which random lipid peroxidation takes place is called autoxidation(19,20). Hydroperoxides are the primary products of autoxidation and once formed, continued oxidation is very likely to take

place, since hydroperoxides decompose to give peroxy radicals, when trace amounts of transition metals are present(21). The mechanism of autoxidation can be represented by the reactior sequence(22), as in Scheme 1.2. (See also Chapter 2, section 2.1.4.)

The most readily oxidized lipids are polyunsaturated fatty acids and esters. Their methylene interrupted double bonds make them particularly good candidates for free radical oxidation, as their bis-allylic hydrogens are easily abstracted to give a delocalized pentadienyl radical:

Scheme 1.3

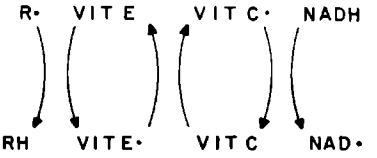


Fortunately living tissues are well protected against peroxide accumulation; the fact that many protective mechanisms exist throughout the tissues would lead to the idea that in the course of evolution lipid peroxidation has been a very real danger.

Vitamin E (α-tocopherol) and vitamin C (ascorbic acid) react rapidly with organic free radicals and it is widely accepted that the antioxidant properties of these compounds are responsible in part for their biological activity. Vitamin E is considerably more lipophilic than vitamin C and in biomembranes, it has been found to be the more potent antioxidant, particularly with respect to lipid peroxidation(12). In pulse radiolysis studies a direct observation of a synergistic interaction(9,25)

between vitamin E and C was reported which resulted in the proposal of the following mechanism of protection against free radicals:

Scheme 1.4



Other oxidation preventing non-enzymic substances have been reported but their mode of action is not as well established. Several enzymes such as glutathione peroxidase have been suggested to protect against lipid peroxidation(26,27) as well.

Random autoxidation of polyunsaturated fatty acids also appears to be an important process in vivo as evidenced by the expiration of pentane and ethane, known fatty acid autoxidation products, by organisms under free radical stress(26,28). The following sources of in vivo initiation have been suggested: halogenated hydrocarbons, ethanol, ionizing radiation, atmospheric pollutants, partly oxidized foods and smoking(3,23,24).

The majority of fatty acids in biological systems occur as phospholipid esters and glycerides which are the structural units in biological membranes. It is assumed that enzymatic hydrolysis of these esters followed by enzymatic oxidation of the resulting fatty acids leads to the physiologically important fatty acid hydroperoxides, hydroxides and prostaglandins. However, recent studies indicate that oxidation products of the phospholipids, themselves, may be important as well. Reincor-

poration of an oxidized fatty acid formed enzymatically into human neutrophil membrane phosphatidylcholines (PC) in vivo, for example, suggests a biological role for PC oxidation products (29-31). Thus it appeared to us that autoxidation of polyunsaturated PC's may very well be an important process, not only because of the potential biological role of the product hydroperoxides but also because of the implications of oxidative degradation of phospholipids in vivo as well as in vitro. We have already mentioned that many diseases are related to lipid oxidation (see also Chapter 2) as well as the aging phenomena. Moreover the oxidative deterioration of food products(32) which has been suggested to produce carcinogens(33) is an important aspect of lipid oxidation as well.

1.2 BIOLOGICAL MEMBRANES

Biological membranes are thin layers of protein and lipid that separate the contents of a single cell or cell component from its environment. They serve as a physical barrier between the inside and outside of the cell or cell component it surrounds, permitting the selective passage of metabolites in and out, but at the same time they are the site of many important enzymic and nonenzymic chemical reactions, such as lipid peroxidation.

Biological membranes consist of a variety of lipids and proteins. The distribution of the different constituents varies from species to species as well as from organ to organ and changes with age(34-36). This is illustrated in Tables 1.1-1.3.

Table 1.1

Protein and Lipid Content of Several Membranes(34)

	Percent of Dry Weight	
	Protein	Lipid
Myelin	18	79
Human erythrocyte	49	43
Bovine retinal rod	51	49
Mitochondria (outer membrane)	52	48
Acholeplasma Laidlawii	58	37
Sarcoplasmic reticulum	67	33
Gram-positive bacteria	75	25
Mitochondria (inner membrane)	76	24

Table 1.2

Lipid Compositions of Some Biological Membranes (expressed as percent by weight of total lipid)(34)

	Human Erythrocyte	Human Myelin
Phosphatidic acid	1.5	0.5
Phosphatidylcholine	19	10
Phosphatidylethanolamine	18	20
Phosphatidylinositol	1	1
Phosphatidylserine	8.5	8.5
Sphingomyelin	17.5	8.5
Glycolipids	10	26
Cholesterol	25	26

Table 1.3

Fatty Acid Composition of Phosphatidylcholine of Skeletal Muscle of Man During Growth(35)

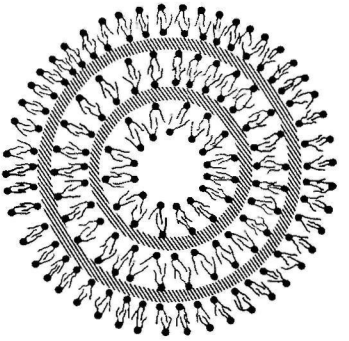
	Fetal age	Postnatal age		
Fatty acid[a]	20 wks	Full term	10 months	16 years
16:0	38.3	35.1	29.0	27.6
16:1ω7	3.9	2.6	1.5	2.0
18:0	7.8	5.1	9.4	9.1
18:1ω9	34.9	23.2	12.5	12.7
18:2ω6	4.7	17.2	37.4	39.3
20:3ω6	0.1	2.6	1.3	1.0
20:4ω6	6.7	10.3	7.9	5.0
22:6ω3	0.7	1.0	0.2	0.7

[a]for explanation of numbering code, see 1.4

The molecular organization of membrane lipids is that of a bilayer, as determined by electron microscopy and X-ray diffraction analysis(34,37). The amphipathic character of the majority of membrane lipids causes them to interact cooperatively with one another in the presence of water. The interaction between lipids can be demonstrated quite simply by shaking a dried membrane lipid extract or purified phospholipid fraction in dilute salt solution(38). The resulting suspension is turbid and a particulate nature can be surmised from its marked light-scattering properties. When suitably stained preparations are examined under the electron microscope, structures consisting of a large number of concentric bilayers similar to myelin membranes are observed. The structures obtained this way are called

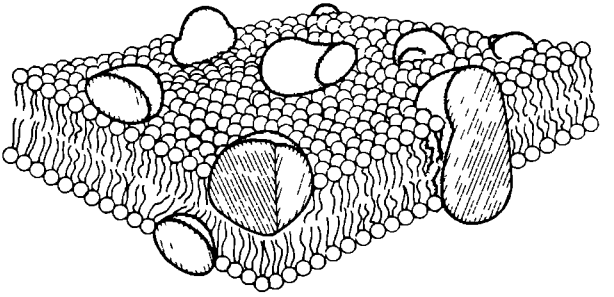
multilamellar vesicles and consist of an estimated 10^9 phospholipid molecules(39). If high-energy ultrasonic radiation is employed to disperse phospholipids in water, the large multilayered structures are broken down to unilamellar vesicles consisting of a single bilayer, which is similar in appearance to the individual layers of the original multilayered structure(37).

Fig. 1.1 Diagrammatic representation of multilamellar vesicles of phosphatidylcholine in excess water, showing only three concentric bilayers.



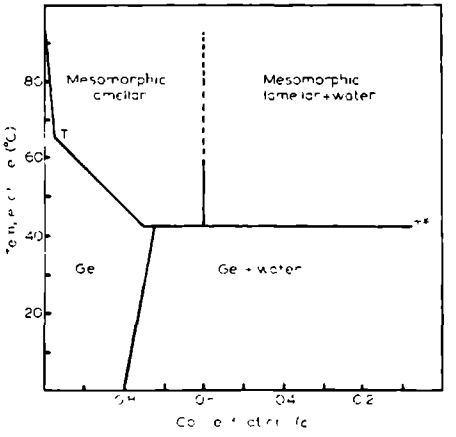
A comparatively recent concept of membrane structure has been developed by Singer and Nicolson(40) to explain the highly mobile character of individual membrane components. The fluid-mosaic model of membrane structure is illustrated in figure 1.2.

Figure 1.2 The fluid-mosaic model of membrane structure proposed by Singer and Nicolson. Globular proteins are interpolated to varying degrees into a phospholipid bilayer and are distributed randomly in the plane of the membrane(40).



The matrix of cell membranes according to this theory is a lipid bilayer to which proteins are either absorbed by predominantly polar forces or they are interpolated into the bilayer in direct contact with the hydrophobic region of the membrane. The structure is thought to be a two dimensional fluid in the sense that individual molecules are able to diffuse readily in the plane of the membrane as evidenced by the lateral motility of membrane lipids and proteins. The fluidity of phospholipid bilayers is dependent on the temperature, the molecular species, and percentage hydration. In Fig 1.3 the phase diagram for the 1,2-di-palmitoyl-L-phosphatidylcholine/water system is shown(41).

Fig. 1.3 Phase diagram of the 1,2-dipalmitoyl-L-phosphatidylcholine/water system(41).



It can be seen that on addition of water the transition temperature (T_t) of the phospholipid is lowered to a certain limiting value (T_t*). This transition temperature is the minimum temperature required for the water to penetrate between the layers of the lipid molecules. Above the T_t line, the phosphatidylcholine/water system exists in a mesomorphic lamellar phase. This phase consists of bimolecular layers of lipid molecules separated by layers of water. The long hydrocarbon chains of the lecithin molecules are in a fluid state and the hydrophilic groups lie on the surface separating the lipid and water layers. On addition of more than 40 wt % water the system dissociates into two phases: the mesomorphic lamellar phase and water. When the phosphatidylcholine/water system is cooled below the T_t line, the hydrocarbon chains adopt an ordered packing. The structure of this phase, the gel, is lamellar as well. The hydrocarbon chains are packed in an hexagonal subcell with the chain axes inclined at 58° to the lipid/water interface. Tilting of the chains may take place so that the glycerol moiety of the molecule can interact favorably with the

water layer. Chapman et al(42) studied the gel-liquid crystal transition for a number of phosphatidyl cholines by infrared spectroscopy, X-ray diffraction and differential scanning calorimetry. Transition temperatures for the gel-liquid crystalline transitions are given in Table 1.4.

Table 1.4

Thermodynamic data for the crystalline→liquid-crystalline transitions of 1,2-diacyl-L-phosphatidylcholines(42)

Acyl chain		Behenoyl 22:0	Stearoyl 18:0	Palmitoyl 16:0	Myristoyl 14:0	Oleoyl 18:1
At maximum hydration	T(°C)	75	58	41	23	22
Monohydrate α-form	T(°C)	-	78	65	51	-
Anhydrous crystalline β-form	T(°C)	120	115	-	-	-

1.3 ESSENTIAL FATTY ACIDS

Essential Fatty Acids is a generic term which historically included all polyunsaturated fatty acids(43). However, it seems preferable to restrict the use of the term to linoleate and its metabolites since they manifest the whole spectrum of PUFA activity. The PUFA's can be divided into a group with 6 carbons beyond the last double bond (ω6, the linoleic acid family) and a group with only 3 carbons beyond the last double bond (ω3, the linolenic acid family). Linoleic acid is converted *in vivo* into the other members of its family, while linolenic acid is the bioprecursor of the linolenic acid family.

Table 1.5

Members of the linoleic acid and linolenic acid families

LINOLEIC ACID FAMILY

Linoleic acid: 9,12-octadecadienoic acid

$C_{18}H_{32}O_2$

$CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_7COOH$

18:2ω6[a]

γ-Linolenic acid: 6,9,12-octadecatrienoic acid

$C_{18}H_{30}O_2$

$CH_3(CH_2)_4CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_4COOH$

18:3ω6

Arachidonic acid: 5,8,11,14-eicosatetraenoic acid

$C_{20}H_{32}O_2$

$CH_3(CH_2)_2CH=CHCH_4CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_3COOH$

20:4ω6

LINOLENIC ACID FAMILY

Linolenic acid: 9,12,15-octadecatrienoic acid

$C_{18}H_{30}O_2$

$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_7COOH$

18:3ω3

No common name: 5,8,11,14,17-eicosapentaenoic acid

$C_{20}H_{30}O_2$

20:5ω3

No common name: 4,7,10,13,16,19-docosahexaenoic acid

$C_{22}H_{32}O_2$

22:6ω3

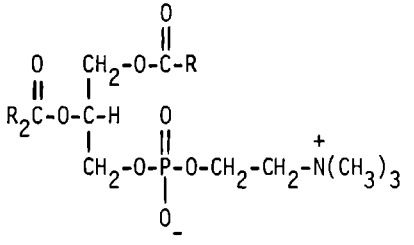
[a]see section 1.4 on nomenclature

The two criteria that determine whether a fatty acid is essential are: 1) weight gains of rats fed PUFA's which previously received a restricted water intake and a PUFA deficient diet; 2) the prevention of dermatitis in animals fed an otherwise fat-free diet.

Only the members of the Linoleic Acid family can be considered essential fatty acids based on both criteria. The Linolenic Acid family does not prevent the dermal symptoms and the fatty acids of this family should therefore not be called essential fatty acids.

1.4 NOMENCLATURE

The abbreviated nomenclature used in this report was derived from the stereospecific numbering system, set forth by the IUPAC-IUD Commission on Biochemical Nomenclature(44). The glycerol derivatives mentioned in this report all have the same stereochemical structure, since all lecithins used were derived from natural sources, i.e., lecithins with the following stereochemical structure:



if R_1 = R_2=palmitoyl: 1,2 dipalmitoyl-sn-glycero-3-phosphorylcholine

1,2 dipalmitoyl-3-sn-phosphatidylcholine

diP-PC

Fatty acids are referred to with their trivial names and listed below with a number code that indicates chain length, number of double bonds and position of the terminal double bond (for example ω6 stands for 6 carbons after terminal double bond):

P Palmitic acid 16:0

$CH_3(CH_2)_{14}COOH$

S Stearic acid 18:0

$CH_3(CH_2)_{16}COOH$

L Linoleic acid 18:2 ω6

$CH_3(CH_2)_4CH{=}CHCH_2CH{=}CH(CH_2)_7COOH$

A Arachidonic acid 20:4 ω6

$CH_3(CH_2)_4CH{=}CHCH_2CH{=}CHCH_2CH{=}CHCH_2CH{=}CH(CH_2)_3COOH$

1.5 SPECIFIC AIM OF THIS STUDY

We have argued that the oxidative metabolism and degradation of polyunsaturated lipids is an important process. In light of the biological role of polyunsaturated lipid oxidation products, it seemed important to study the autoxidation of these lipids and chemically identify the products. Thus we studied the autoxidation of linoleic acid, arachidonic acid, as well as linoleic and arachidonic acid containing lecithins, and identified the primary products of oxidation, the hydroperoxides. The lecithin autoxidation was carried out in aqueous emulsion, since under the conditions used, lecithins form liposomes having a bilayer structure, which physically resembles biological membranes. In order to determine how other natural membrane constituents effect lecithin autoxidation, we studied the

product distribution of lecithin hydroperoxides, formed in the cooxidation of diL-PC with steroids (cholesterol and 7-dehydrocholesterol), vitamin E (α-tocopherol), free fatty acid (linoleic acid) and a saturated lecithin (diP-PC). Similarly, we studied the cooxidation of 1S,2A-PC with α-tocopherol.

1.6 CONTENTS OF THE DISSERTATION

In chapter one an introduction is given on lipid peroxidation, essential fatty acids, and the molecular arrangement of lecithins (PC's) in biological membranes. A table with the natural occurrence of some lipids is given as well as the nomenclature of lecithins, and fatty acids.

In chapter two the analysis of the primary autoxidation products of linoleic acid and arachidonic acid is reported. Four linoleic acid hydroperoxides and 6 arachidonic acid hydroperoxides were analyzed and their structure identified.

In chapter three a literature review on product studies of monolayer and bilayer oxidations as well as on HPLC separations of lecithins is presented. The autoxidation of synthetic polyunsaturated lecithins (1P,2L-PC and 1S,2A-PC) and egg lecithin is described as well as the analysis of the product hydroperoxides. Reverse phase separation was found to be the method of choice in the isolation of the lecithin hydroperoxides; subsequent hydrolysis led to the corresponding fatty acid hydro(pero)xides which could be analyzed by normal phase HPLC.

In chapter 4 the mechanism of linoleate autoxidation is extensively discussed and related to the product distribution of the hydroperoxides formed in the autoxidation of lipid mixtures resembling biological

membranes. The results of cooxidations of diL-PC with diP-PC, cholesterol, 7-dehydrocholesterol, linoleic acid and α-tocopherol are given and the physical and chemical implications discussed. The consequences of the results of the cooxidation with α-tocopherol for the fate of a lipid hydroperoxide in lipid membranes is discussed with respect to the peroxyl radical expulsion hypothesis.

In chapter 5 an extensive treatise on the mechanism of arachidonate autoxidation is given. The results of bilayer arachidonate autoxidation with and without α-tocopherol present are discussed and compared with bulk phase autoxidation. The product mixture of the cooxidation of 1S,2A-PC and α-tocopherol in aqueous emulsion surprisingly lacked the 5-HPETE. Possible consequences for physiological oxidations are suggested.

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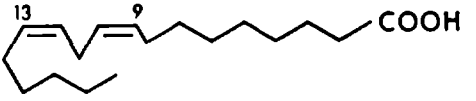
CHAPTER 2

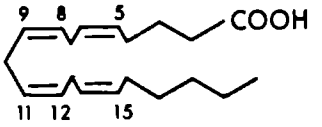
AUTOXIDATION OF POLYUNSATURATED FATTY ACIDS
FORMATION AND ANALYSIS OF LINOLEIC (18:2) AND ARACHIDONIC (20:4) ACID HYDROPEROXIDES

2.1 INTRODUCTION

2.1.1 IMPORTANCE OF PUFA OXIDATION STUDIES



Linoleic Acid (18:2 ω6); LA



Arachidonic Acid (20:4 ω6); AA

This chapter will deal with the autoxidation of linoleic acid (18:2) and arachidonic acid (20:4). The air oxidation of these two Polyunsaturated Fatty Acids (PUFA) was considered important for the following reasons:

1) the degradation of fats and other lipids *in vitro* and *in vivo* takes place by oxidation processes;

2) the synthesis of biologically important lipid hydroperoxides via the autoxidation pathway was found to be convenient and versatile;

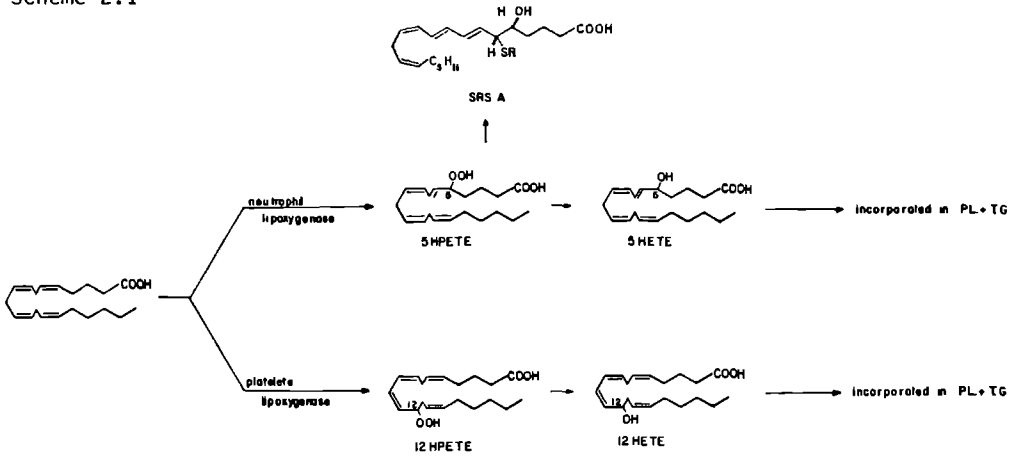
3) fatty acid autoxidation will provide a model study for the more complicated phosphatidylcholines (Chapter 3).

The choice of the two polyunsaturated fatty acids for this study can be rationalized as follows. LA possesses two double bonds and can be expected to give only four hydroperoxides upon autoxidation. H atom abstraction from the bisallylic C-11 results in the formation of a pentadienyl radical which traps oxygen to give conjugated diene peroxy radicals and eventually the corresponding hydroperoxides. Previous studies on methyllinoleate autoxidation have shown that only t,t and t,c stereoisomers are formed with the trans double bond always adjacent to the oxygen substitution(1,2). Information gained from the relatively simple LA autoxidation can help clarify the more complicated product mixture expected from AA autoxidation. AA is precursor to many physiologically active metabolites. The biological importance of the AA and LA oxidation products studied here (hydroperoxides), will be discussed in the following section.

2.1.2. BIOLOGICAL IMPORTANCE OF LA AND AA HYDROPEROXIDES

Enzymes capable of synthesizing specific hydroperoxides from polyunsaturated fatty acids are widely present in nature. The most important class of hydroperoxide generating enzymes are lipoxygenases which catalyze the formation of t,c diene hydroperoxides from c,c homoconjugated fatty acids(3,4). No plant species has been found so far that does not have a lipoxygenase enzyme present. It is therefore most surprising that a physiological role for plant lipoxygenases and their product hydroperoxides has yet to be established.

Scheme 2.1



This same class of enzymes is widely present in human beings and other mammals as well. A variety of lipoxygenase enzymes convert arachidonic acid into hydroperoxides with important biological functions. For

instance, a lipoxygenase present in human neutrophils converts AA into 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid (5-HPETE) and 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE). These oxygenated arachidonic acid metabolites were suggested to be intermediates in the biosynthesis of the Slow Reacting Substance of Anaphylaxis (SRS-A)(5). SRS-A is a prolonged smooth muscle contracter that is not inhibited by antihistamines and is thought to play a role in respiratory ailments such as asthma(6-8). This same AA oxidation pathway seems to be responsible for another physiological event in human neutrophils: It was shown that chemotactic factors, arachidonic acid and leukocyte-derived lipoxygenase products of arachidonate (5-HPETE, 5-HETE, and 5,12-dihydroxyeicosatetraenoic acids) stimulated stereospecific uptake of ^{3}H-2-deoxyglucose by human neutrophils. However, of all stimuli tested only the effects of 5-HETE were not inhibited by 5,8,11,14-eicosatetraynoic acid at concentrations which inhibit release of arachidonate metabolites from leukocytes. The data suggest that 5-HETE mediates the coupling of membrane receptor stimulation to the functional response of hexose uptake in human neutrophils(9). It was suggested that AA metabolism could be associated with stimulation of hexose transport in insulin sensitive tissue as well(10).

12-HPETE and 12-HETE, which are formed in human platelets, exhibit chemotactic and chemokinetic activity for neutrophils and eosinophils, a process which is involved in inflammation(11). Both 12- and 5-HETE are incorporated into phospholipids and triglycerides and are suggested to effect membrane structure and function(12,13). Stimulation of hexose uptake in human neutrophils by 5-HETE and reincorporation of this chemotactic arachidonic acid metabolite into phospholipids and triglycerides suggests to us that 5-HETE containing phospholipids and

triglycerides may be directly responsible for hexose uptake. Synthesis and pharmacological testing of a 5-HETE lecithin is therefore called for.

Another enzyme that forms hydroperoxides from polyunsaturated fatty acids is cyclooxygenase. This enzyme is mainly known to be responsible for the biosynthesis of PGG_2 from AA(14). However, it was also found to form a 77:23 ratio of 9 and 13 hydroxyoctadecadienoic acid when incubated with linoleic acid(15).

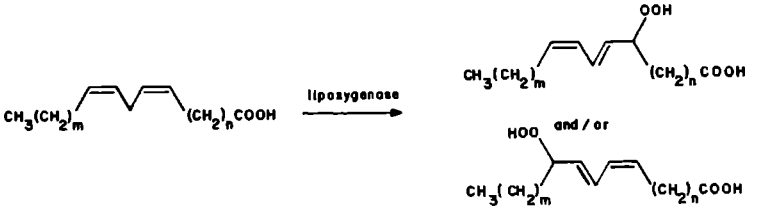
Similarly LA and AA metabolism in VX_2 carcinoma tissue suggests the formation of 13 and 9 hydroxyoctadecadienoic acid and 15 and 11 HETE from cyclooxygenase(16). The formation of these fatty acid metabolites was inhibited by indomethacin, and PGE_2 was the major product of AA metabolism, suggesting a cyclooxygenase pathway for this conversion.

Lipid hydroperoxides in general appear to have physiological effects; it was suggested that they enhance anaphylactic release of histamine and SRS-A, based on such effects found for 13HP-LA and 15 HPETE(17). Furthermore, both linoleic acid and arachidonic acid hydroperoxides have been found to inhibit the formation of PGI_2(18), an important protaglandin which is known to inhibit platelet aggregation. Linoleic acid hydroperoxides and arachidonic acid hydroperoxides stimulate aggregation which is in agreement with the observation that high concentrations of hydroperoxides are present in rabbit platelets in the initial stages of the development of thrombosis induced by ADP, adrenalin, serotonin, and thrombin(19).

2.1.3. SYNTHETIC METHODS FOR LINOLEIC ACID AND ARACHIDONIC ACID HYDROPEROXIDES

A variety of enzymatic pathways lead to single lipid hydroperoxides, from c,c homoconjugated polyunsaturated fatty acids. The product hydroperoxides are single enantiomers that have a t,c conjugated diene.

Scheme 2.2



For example soybean Lipoxygenase-type 1 leads mainly to (ω-6)-L_S-hydroperoxy fatty acid. Thus the 13 hydroperoxy linoleate and 15 hydroperoxy arachidonate (15-HPETE) are available via this enzyme(1). Porter, et al. established procedures for preparing linolenic and arachidonic acid hydroperoxides by incubating the fatty acid with commercial soybean Lipoxygenase enzyme and subsequent HPLC purification of the oxidized product(20). Similarly, potato lipoxygenase was found to specifically produce the 9-D-hydroperoxylinoleate from linoleic acid and the 5-D-HPETE from arachidonic acid(21). However, decomposition of the hydroperoxides due to impurity of the enzyme is often difficult to prevent. Lipoxygenases(22) and cyclooxygenase(11) have been found to form

hydroperoxides other than the ones described here, however, they are only partially product specific or available only in limited quantities.

Singlet oxygen oxidation of arachidonic acid has been shown to be a convenient route for the synthesis of eight HPETE isomers(23). Unfortunately the 15,14 and 5,6 isomer pairs are not separable, and therefore only the 12,11,9 and 8 isomers can be isolated pure.

An organic synthetic method of certain HETE isomers was recently reported(21,24). The 5,11,14 and 15-HETE isomers each were synthesized via individual routes. A two step low temperature synthesis of the corresponding HPETE's from these HETE isomers was also reported. Quite recently a novel organic synthesis of PUFA hydroperoxides and hydroxides has been reported(25). This elegant synthesis based on Ag^+ ion assisted vinylcyclopropyl bromide ring opening reaction presents the potential to synthesize a wide variety of fatty acid hydroperoxides, hydroxides and other derivatives.

The most convenient synthesis of tc and tt diene hydroperoxides seems to be autoxidation of PUFA's followed by HPLC separation. Chan, et al. reported on the autoxidation of methyllinoleate followed by product isolation by HPLC(1). This chapter will present the results of the autoxidation of linoleic acid, arachidonic acid and methylarachidonate. The product hydroperoxides were isolated by HPLC and reduced to the corresponding hydroxides. Structure determination was based on GC-MS, UV and IR.

2.1.4 AUTOXIDATION OF LIPIDS

Organic materials are not thermodynamically stable in oxygen, but are spontaneously converted to oxidized products. This process is called auto-oxidation or autoxidation. Fortunately these reactions are kinetically slow or life as we know it would be impossible(26). The autoxidation of lipids is a free radical chain process which in homogeneous solution at partial oxygen pressure above ca 200 torr can be represented by the following reaction sequence(27-35).

Scheme 2.3

Initiation:	In_2	$\rightarrow$ $2In\bullet$	[1]
	$In\bullet + RH$	$\rightarrow$ $InH + R\bullet$	[2]
Propagation:	$R\bullet + O_2$	$\rightarrow$ $ROO\bullet$ (fast)	[3]
	$ROO\bullet + RH$	$\rightarrow$ $ROOH + R\bullet$ (slow)	[4]
	$ROO\bullet + RH$	$\rightarrow$ $ROO\overset{\bullet}{R}H$ (cyclic)	[4a]
Termination:	$ROO\bullet + ROO\bullet$	$\rightarrow$ non-radical organic products + O_2	[5]

In this scheme RH represents a lipid (or other organic substrate), R• the carbon centered radical that is generated when RH reacts with peroxy radical ROO•. Addition of oxygen to an alkyl radical (3) is extremely fast and in many cases probably diffusion controlled, i.e., $k_3 \approx 10^9\ M^{-1}\ s^{-1}$(31).

This means that the rate of oxidation above about 100 torr is independent of the oxygen pressure, that the rate controlling propagation step involves reaction of a peroxyl radical with the substrate, and that the rate controlling termination step involves the reaction of two peroxyls to give non radical products(34). The rate of oxygen consumption for a given system which follows the reactions (1)-(5) can be represented by equation 6:

$$\frac{-d[O_2]}{dt} = \frac{k_p[RH]R_i^{1/2}}{(2k_t)^{1/2}} \qquad \text{(eq. 2.1)}$$

where R_i is the rate of initiation, k_p the rate constant for the rate controlling chain propagation and k_t the rate constant for chain termination. The rate constant ratio $k_p/(2k_t)^{1/2}$ is generally referred to as the oxidizability of the substrate.

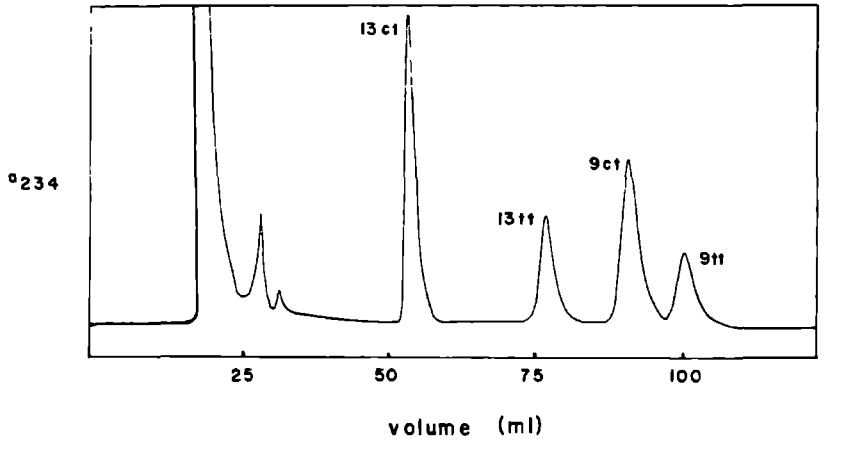
Polyunsaturated fatty acids are among the most oxidizable substrates in nature. Their bisallylic hydrogens are easily abstracted to give stabilized delocalized pentadienyl radicals, which then trap oxygen and are converted to a hydroperoxide upon H atom abstraction (eq (3)-(4)). In vivo a radical chain reaction such as lipid autoxidation is thought to be initiated by any of the following sources: superoxide anion ($O_2^{\cdot}$), ozone (O_3), NO_2, radiation, halogenated hydrocarbons such as CCl_4, halothane etc., ethanol poisoning, transition metals, partially oxidized foods, etc.(26,36) Although termination reactions <u>in vivo</u> have not been studied

in detail it is presumed that inhibitors such as vitamin E and C and thiols scavenge radicals and convert them to unreactive species.

2.2 RESULTS

Air oxidation of neat linoleic acid, arachidonic acid and methyl arachidonate is self-initiated and occurs rapidly. After 48-72 hr, an estimated 15-25% of reactant had been oxidized and the reaction was stopped to prevent excessive decomposition. The products were UV and peroxide positive on TLC, and could be analyzed by HPLC.

Fig. 2.1 HPLC of linoleic acid alcohols derived from triphenyl phosphine reduction of autoxidized linoleic acid. Product detection by uv at 234 nm. Position of oxygen substitution and stereochemistry is indicated for each identified peak. Solvent: 1/16/983, acetic acid/isopropanol/hexane.



Only the 9 tc and 9 tt linoleic acid hydroperoxides as well as the 9 and 8-HPETE methyl esters could not be separated. The autoxidation products were then reduced by triphenyl phosphine to give the corresponding alcohols. The HPLC separation of the hydroxy compounds was satisfactory (see Fig. 2.1 and 2.2), only the 9 and 8 methyl hydroxy arachidonates were not separated.

Figure 2.2 HPLC of HETE methyl esters derived from NaBH4 reduction of autoxidized methyl arachidonate. Product detection by UV at 234 nm. Position of oxygen substitution is indicated for each identified peak. Solvent 5/995, isopropanol/hexane.

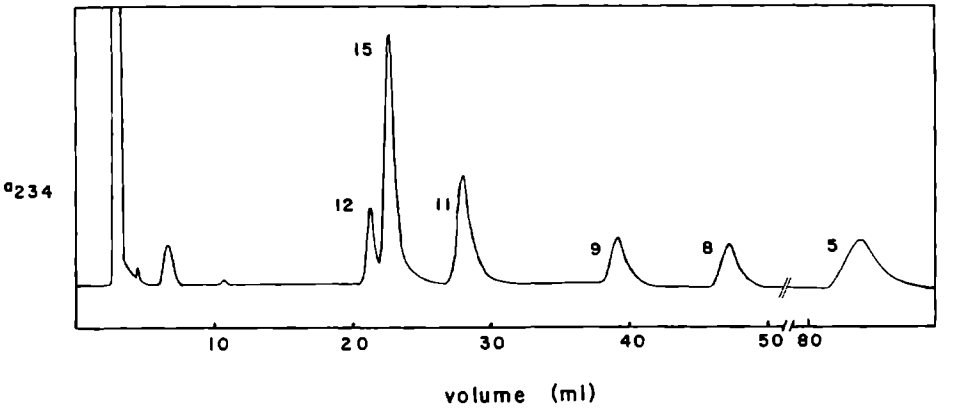
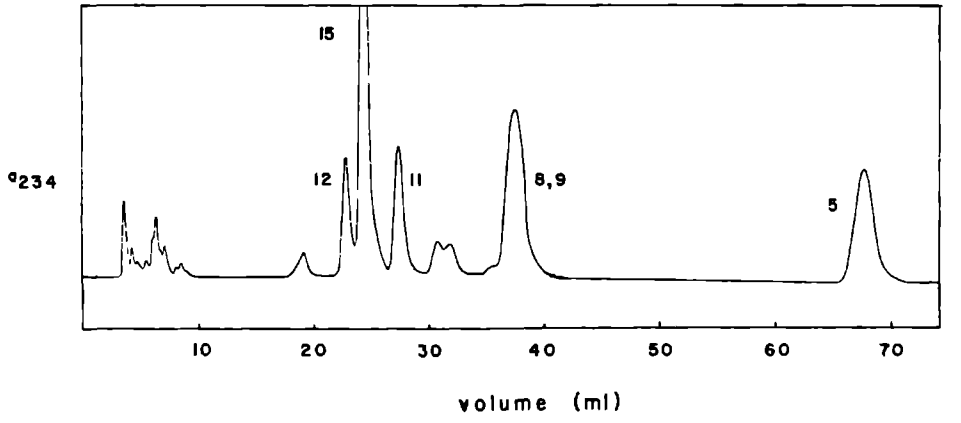


Fig. 2.3 HPLC of HPETE's formed in autoxidation of arachidonic acid. Product detection by UV at 234 nm. Position of oxygen substitution is indicated for each identified peak. Solvent 1/10/989, acetic acid/isopropanol/hexane.



The linoleic acid alcohols were isolated by HPLC and converted to the methyl esters with diazomethane. The hydroxy methyl linoleates obtained this way were hydrogenated, silylated, and analyzed by gc/ms to determine the position of the hydroxy substitution (see table 2.1)(37).

Table 2.1

Products Isolated from HPLC Separation of Linoleic Acid Oxidation after $P\emptyset_3$ Reduction

Retention volume(ml)	Principal mass spec fragments[a]	structure of hydroxy-octadecadienoic acid
54	173,315	13 trans,cis
76	173,315	13 trans,trans
86	229,259	9 trans,cis
100	229,259	9 trans,trans

[a]Mass spectral fragments are from the hydrogenated and trimethylsilylated methyl esters.

Trans,cis and trans,trans substituted dienes were identified by ir and uv spectroscopy. Thus, the trans,cis substituted dienes absorbed at 985 and 950 cm^{-1} in the infrared, whereas the trans,trans compounds showed absorption at 989 cm^{-1}(1). The trans,cis substituted dienes absorbed in the ultraviolet at 236.0 nm and the trans,trans compounds at 232.5. Thus it was found that the four linoleic acid hydroperoxides formed as well as the corresponding hydroxides eluted in the same order as the methyl linoleate oxidation products: 1. 13 t,c; 2. 13 t,t; 3. 9 t,c; 4. 9-t,t-hydro(pero)xy octadecadienoic acid. This was confirmed by isolating the hydroxy linoleic acid isomers by HPLC, and subsequent injecting the methylated individual fractions: the order in which the oxygenated linoleic acid isomers were eluted was the same as for the methyl esters.

Similarly the hydroxy methyl arachidonates were analyzed by GC/MS, uv, ir and by comparison with peroxide products formed from singlet oxygen oxidation of methyl arachidonate(23). The structure assignments are presented in Table 2.2.

Table 2.2

Products Isolated from HPLC Separation of Methyl Arachidonate Oxidation

Retention volume(ml)	principal mass spec fragments[a]	structure of methyl ester eicosatetraenoic alcohols
22	301,215	trans,cis-12
25	343,173	trans,cis-15
27	287,229	trans,cis-11
37	259,257,245,271	trans,cis-9,8
68	203,313	trans,cis-5

[a]Mass spectral fragments are from the hydrogenated and trimethylsilylated methyl esters.

Oxidation of arachidonic acid proceeds in much the same manner as the methyl ester oxidation. The results are shown in Table 2.3. The structure assignments were based on comparison with $^1O_2$ oxidation products of Arachidonic acid and conversion of hydroperoxides to the hydroxy methyl esters.

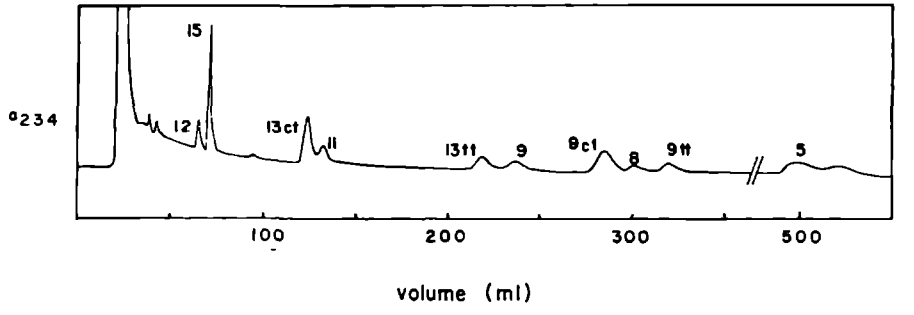
Table 2.3

Products Isolated from HPLC Separation of Arachidonic Acid Oxidation

Retention volume(ml)	Structure of hydroperoxy eicosatetraenoic acid
21	trans,cis-12
23	trans,cis-15
28	trans,cis-11
39	trans,cis-9
47	trans,cis-8
83	trans,cis-5

The HPLC separation of a mixture of linoleic acid alcohols and arachidonic acid alcohols (HETE's) was found to give complete resolution of all alcohols formed after reduction of the autoxidized fatty acids (fig. 2.4). Normal phase HPLC separation may therefore be useful in analyzing oxidation products of complex fatty acid mixtures.

Fig. 2.4 HPLC of mixture of linoleic acid alcohols and arachidonic acid alcohols obtained by reduction of the autoxidized fatty acids. Product detection by UV at 234 nm. Position of oxygen substitution and the stereochemistry of the linoleic acid alcohols are indicated for each identified peak. Solvent: 1/7/992, acetic acid/isopropanol/hexane.

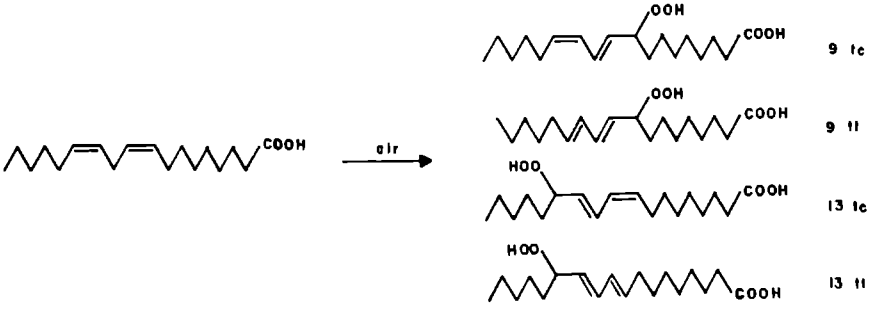


2.3 DISCUSSION

Linoleic acid autoxidation

Air oxidation of neat linoleic acid results in the formation of 9-t,c, 9-t,t, 13-t,c, and 13-t,t hydroperoxides.

Scheme 2.4



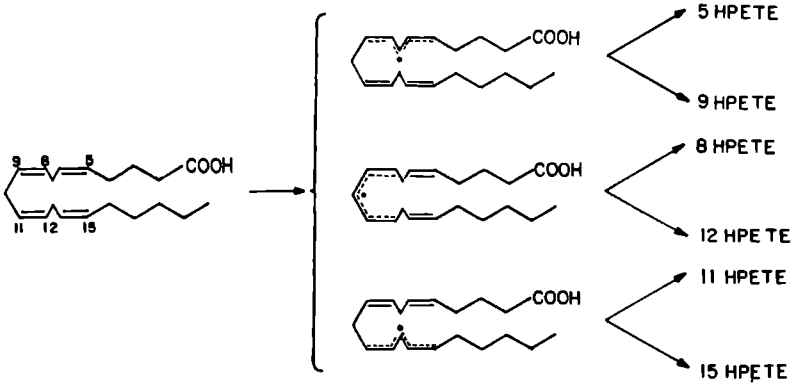
The four products formed can be analyzed by HPLC, but complete resolution is achieved only after reduction of the hydroperoxides to the corresponding hydroxides. Thus, when analyzing linoleic acid oxidation mixtures, the procedure of reducing the hydroperoxides to the hydroxides is the method of choice, since not only do the alcohols give complete resolution by HPLC, but also because the alcohols unlike the hydroperoxides do not readily isomerize(38). The chromatographic behavior of the linoleic acid hydroperoxides and hydroxides as well as the methyl linoleate hydroperoxides and hydroxides are very much analogous: the 13 isomer is eluted before the 9, and the t,c isomer before t,t isomer in both cases.

The autoxidation of neat linoleic acid at room temperature resulted in the formation of predominantly t,c hydroperoxides, the t,c/t,t ratio being approximately 2:1. The 9 and 13 isomers were present in equal amounts both for the t,c and t,t isomers. Methyl linoleate gave the same results under the conditions used. Concentration and temperature dependence of the product formation will be further discussed in Chapter 4.

AA autoxidation

AA autoxidation is similar to LA oxidation in a qualitative way, but dramatically different in a quantitative way. Analogous to the LA autoxidation, only those positional isomers are formed that are generated from O_2 entrapment by a delocalized pentadienyl radical. Thus hydrogen abstraction at center 7 gives rise to the 5 and 9 HPETE; similarly, abstraction of hydrogens at positions 10 and 13 will lead respectively to the 8,12 and 11,15 isomer pairs.

Scheme 2.5



Other C-H bonds in the molecule are much less susceptible to radical attack and products derived from such a process are not formed in any appreciable amount. The predominant hydroperoxides formed have the trans,cis substituted diene unit, with the trans,trans products formed in very small amounts. In fact all of the major products that we have isolated from the free acid oxidation have the trans,cis diene structure. It seems likely, however, that the trans,trans compounds are present to some extent. It is obvious from the HPLC chromatograms in Figs. 2.1-2.3 that the arachidonic acid autoxidation gives relatively more trans,cis products when compared with linoleic acid oxidation. Also it is shown in this study that the positional isomers of AA autoxidation unlike the LA autoxidation are not present in equal amounts. The 15- and 5-HPETE are the predominant products formed under these circumstances, while the other isomers are generated to a lesser extent and are present in about equal amounts. The implications of these observations are further discussed in Chapter 5.

Isolation of Arachidonic Acid Oxidation Products

Both individual hydroperoxides and hydroxides of arachidonic acid and its methyl ester can be isolated by the method reported here. No complex organic synthetic effort is required, and mg quantities of the isomers can be isolated readily. The method described here can be applied for large scale preparative HPLC isolation of the 5-HPETE, SRS-A precursor.

Air oxidation is to be preferred over singlet oxygen oxidation of arachidonic acid, when the 5 or 15 isomer are to be isolated. In the reaction of singlet oxygen with arachidonic acid, the 6 and 14 non-conjugated isomers are formed in addition to the 5,8,9,11,12 and 15 substituted compounds. By chance the 6,5 and 15,14 isomer pairs

co-chromatograph, and singlet oxygen is thus not preparatively useful for the 5 and 15 isomers.

We have modified the method described here by using a 5 μ column and have been able to separate all HETE's formed from the air oxidation of arachidonic acid and subsequent triphenylphosphine reduction. However, under those conditions the 9 and 8 HETE methyl esters were still only partially separable. It should also be noted that hydroperoxides formed in the autoxidation of lipids are racemic mixtures and the method described here can therefore not be applied for the preparation of individual enantiomers.

2.4 EXPERIMENTAL

General Remarks

1) ^{1}H NMR spectra were determined on a JOEL JNM-100 at 100 MHz, a Bruker NR80 at 80 MHz or a Bruker WH-250 FT spectrometer at 250 MHz.

2) IR spectra were taken on a Perkin Elmer 297 spectrophotometer.

3) UV spectra were taken on a Perkin-Elmer 576 ST spectrophotometer.

4) GC-MS spectra were recorded with a Hewlett-Packard 5992A GC-MS system.

5) High Performance Liquid Chromatography was conducted with Waters Associates Model 6000A.

6) Differential Scanning Calorimetry (DSC) was carried out on a Perkin-Elmer DSC I b.

7) A Nikon binocular microscope with phase contrast attachments and a Polaroid camera were used for the microscope studies.

8) A Hewlett Packard HP 5830A gas chromatograph was used with a 3 ft 3% OV 101 0.1% Carbowax column for analysis of volatile fatty acid derivatives.

Fatty acids (99%+) were obtained from NuChekPrep (Elysian, MN) and used without further purification.

TLC: Silica gel 60F-254, 5 cm x 20 cm x 0.25 mm precoated plates were used (Merck and Co., St. Louis, MO). The solvent used for elution of the fatty acid derivatives was acetic acid:isopropanol:hexane (1/20/229, vol/vol). Peroxides were visualized by ferrous thiocyanate spray.

HPLC: A Waters Associates 10 μ-porasil column (7.8 mm I.D. x 30 cm) was used for separation of the fatty acid derivatives. Hexane and isopropanol were distilled before use. The solvent systems used were: acetic acid:isopropanol:hexane, 1/10/989, vol/vol for the arachidonic acid hydroperoxides; isopropanol:hexane, 5/996, vol/vol for the hydroxy methyl arachidonates; and acetic acid:isopropanol:hexane, 1/16/983, vol/vol for the linoleic acid hydroxides.

Autoxidation of the fatty acids: Arachidonic acid and linoleic acid (99%+, NuChekPrep, Elysian, Minnesota) were used without further purification. The fatty acid was taken up in dry ether and this solution was placed in a round-bottomed flask. The ether was removed in vacuo leaving the arachidonic acid as a film coating the wall of the flask. Air which had been cleaned and dried by sulfuric acid, KOH, and calcium sulphate was passed through the flask at room temperature. The oxidation could be followed by thin layer chromatograhy, peroxide and uv positive products being observed at R_f values between .05-.25. After 48 hr, the oxidation was stopped and the residue was taken up in dry hexane and subjected to HPLC.

Reduction of Hydroperoxides with Triphenylphosphine: The hydroperoxides generated by autoxidation were taken up in ethyl ether that had been previously saturated with water and excess triphenyl phosphine was added.

The solution was magnetically stirred at 0°C for 1/2 hr and the products were then purified by HPLC.

Gas Chromatography-Mass Spectrometry: The isolated hydroxy fatty acid methyl esters were characterized by GC-MS after hydrogenation over PtO_2 and trimethylsilation with N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA)(37). Gas chromatography mass spectrometry (OV 101, 200-230°) led to a peak detected at approximately six minutes. The characteristic fragmentation alpha to the trimethylsiloxy group indicated the position of oxygen substitution on the fatty acid chain.

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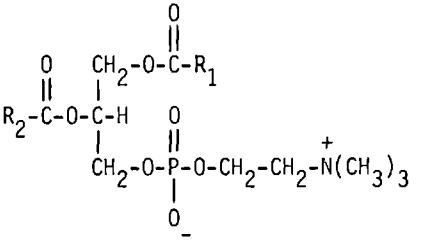
CHAPTER 3

AUTOXIDATION OF POLYUNSATURATED LECITHINS: ANALYSIS OF PRODUCT HYDROPEROXIDES

3.1 INTRODUCTION

3.1.1 BIOLOGICAL IMPORTANCE OF PHOSPHATIDYLCHOLINE OXIDATION

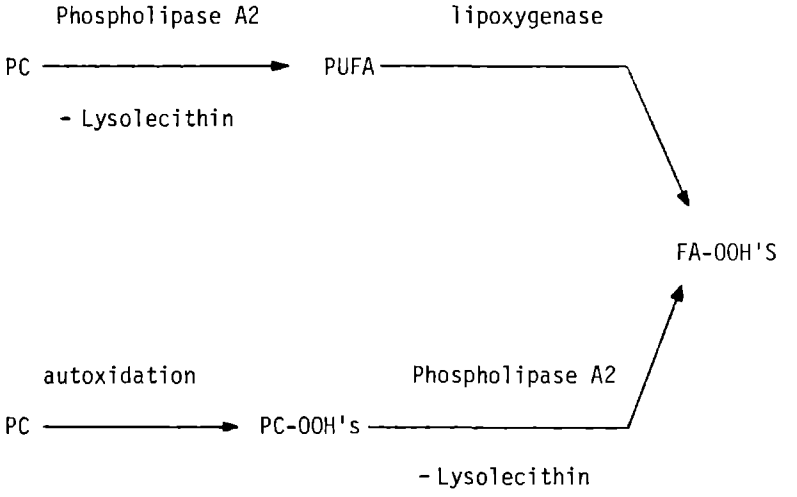


Phosphatidyl Choline

Lecithin

Fatty acid peroxidation leads to the biologically important fatty acid hydroperoxides and hydroxides (see Chapter 2). Enzymatic hydrolysis of phospholipids by Phospholipase A_2 has been suggested to initiate the formation of the fatty acid hydroperoxides(1). It has been assumed that enzymatic oxidation takes place after the Phospholipase hydrolysis, since an important class of oxidative enzymes, the lipoxygenases, have not been found to be effective for phospholipids. However, since we have found that phospholipase A-2 hydrolyzes phosphatidylcholine hydroperoxides and hydroxides in addition to the unoxidized lecithins, it seems conceivable that nonenzymatic oxidation of phospholipids followed by phospholipase hydrolysis is an alternative route to fatty acid hydroperoxides and hydroxides.

Scheme 3.1

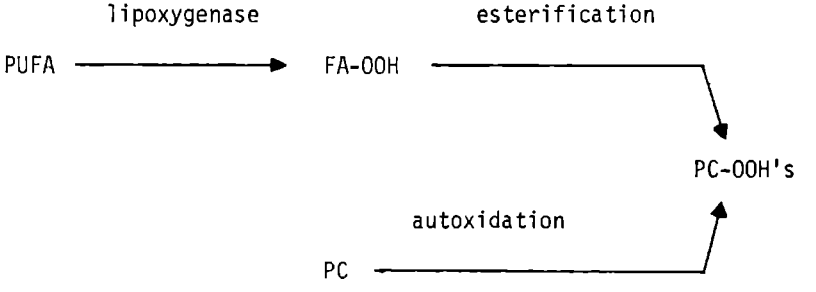


It has been demonstrated that in vivo autoxidation of polyunsaturated fatty acids containing lipids is an important process, as evidenced by the expiration of pentane and ethane, (Scheme 3.2) known fatty acid oxidation products, by organisms under free radical stress(2). Thus autoxidation of polyunsaturated fatty esters such as lecithins may in certain cases be the first step in the generation of fatty acid hydroperoxides and hydroxides.

Scheme 3.2

Membrane damage induced by radiation(3), halogenated hydrocarbons(2,4) or ethanol poisoning(5) has been proposed to be the result of phospholipid destruction by molecular oxygen. In fact a theory of aging has been proposed that rests in part on the free radical oxidation of membrane lipids(6). Moreover lecithin hydroperoxides generated in an autoxidation process appear to have a specific biological function as well. Re-incorporation of enzymatically oxidized fatty acids into phosphatidylcholines in vivo suggests that PC hydroperoxides are directly involved in a physiological process(7). Both the 5 and 12 HETE are esterified into cellular triacylglycerols and phospholipids in human neutrophils (12 and 5 HETE) and in granulocytes (5 HETE). It has been suggested that the re-esterification of these hydroxy fatty acids into membrane lipids may be one mechanism for controlling such functions as degranulation and chemotaxis(7).

Scheme 3.3

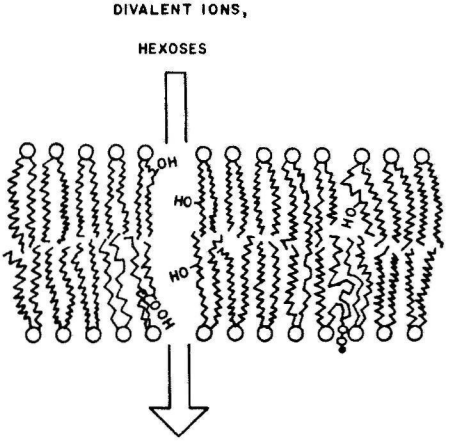


A possible mechanism for such a physiological role for the hydroxy PC's and triacylglycerols may be a change in the permeability of these

oxidized membrane components for electrolytes(8) or other polar species such as sugars(9). Kargan, et al.(10) investigated the Ca++ permeability of sarcoplasmic reticulum membranes, and they found that only phospholipid hydroperoxides but not fatty acid hydroperoxides increased Ca^{++} permeability in these membranes. Volpi, et al.(8) suggest that an AA dependent lipoxygenase mediated pathway is responsible for an increase in the permeability of rabbit neutrophil plasma membrane for specifically Ca^{++} ions.

In a crystalline structure hydroxy fatty acids are arranged in a way that is mainly defined by the H bonding between the hydroxyl groups. This causes the formation of clusters of hydroxy fatty acids, both in the crystalline structure as well as in monolayers(11). It seems conceivable to us that hydroxy or hydroperoxy-fatty acids and/or phospholipids, generated in a biological membrane form such a cluster, providing a tunnel for polar molecular species in an otherwise apolar inner region of a membrane bilayer (Fig. 3.1).

Fig. 3.1 Proposed Mechanism for Increased Permeability of PC Bilayer for Polar Species



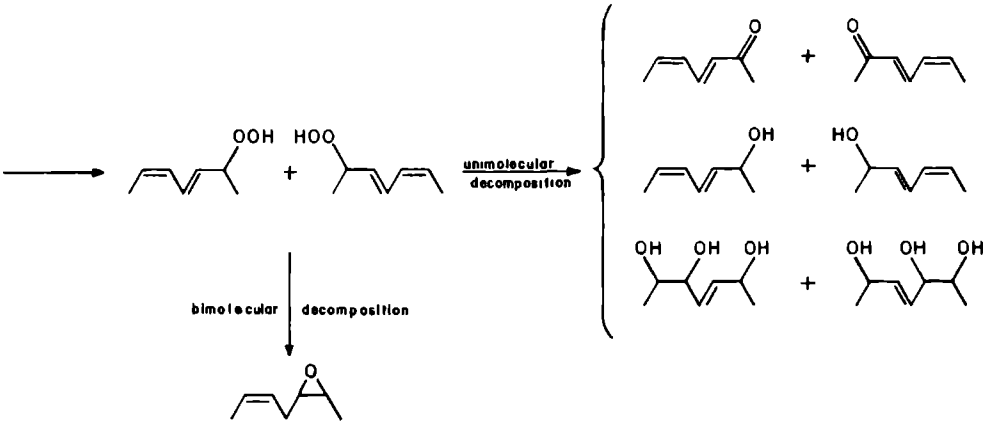
Thus enzyme controlled oxidation or uncontrolled autoxidation leads to the formation of polar lipids which may serve as endogenous ionophores for divalent cations and which may affect hexose transport. Specific Phosphatidylcholine hydroperoxide isomers are presently being synthesized to further investigate this phenomena. Thus the autoxidation of lecithins appears to be an important process for several reasons:

1) PC hydroperoxides are potentially important biological compounds; autoxidation provides a (bio)synthetic route towards this class of compounds.
2) Autoxidation is a degradative pathway of lipids both in vivo and in vitro; it is particularly important under conditions of free radical stress.

3.1.2 PRODUCT STUDIES IN BILAYER AND MONOLAYER OXIDATIONS

Sessa, et al. reported on the structures of bitter tasting lecithins isolated from soybean flakes(12). The main oxygenated fatty acids analyzed were epoxides, α,β-unsaturated ketones, α,β-unsaturated alcohols and trihydroxides as well as a variety of minor products (Scheme 3.4). Except for the epoxy compounds, the oxygenated fatty acids were similar to the products formed by homolytic decomposition of linoleic acid hydroperoxide. The epoxide formation, the authors suggest, may have occurred by the action of fatty acid hydroperoxides on an unsaturated fatty acid. Thus all products identified were generated from the primary products of oxidation, the hydroperoxides.

Scheme 3.4



Mead, et al.(13) carried out the autoxidation of fatty acid monolayers adsorbed on silica gel. Linoleic acid produced predominantly 9,10 epoxy and 12,13 epoxyoctadecenoic acid in roughly equal quantities, diene products being present in relatively small amounts. Oleic acid (16:1) produced no detectable amount of epoxide. They suggest that peroxy radical attack on an unoxidized linoleic acid molecule is responsible for epoxide formation.

Crawford, et al.(14) reported on the autoxidation of diLPC isolated from soybeans almost simultaneously with the publication of the results described in this chapter. The authors of this paper isolated diLPC from soybeans by RP-HPLC and after subsequent autoxidation in aqueous emulsion, separated and purified the mono and dioxidized lecithins. The two fractions were analyzed by mass spectrometry after reduction,

transmethylation, and silylation. Both fractions contained only 9 and 13 hydroxy dienes, no epoxides could be detected. This is in agreement with the product analysis of autoxidized synthetic diLPC, as described in this chapter.

Recently, Terao, et al.(15) studied the oxidation of soybean PC and dilinoleic PC (diLPC) by singlet oxygen ($^1O_2$) using a methylene blue sensitized photo oxidation system. The oxidation products, mono and dihydroperoxides, were converted to saturated diglyceride derivatives upon treatment with phospholipase C and hydrogenation. GC-MS analysis of the saturated diglycerides was performed after silylation. However, the position of the oxygenation could only be determined after hydrolysis, hydrogenation, methanolysis and silylation. The ratio of the 9,10,12 and 13 isomers formed from the $^1O_2$ oxidation of linoleate was 34:16:16:34.

3.1.3 HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATIONS OF PHOSPHOLIPIDS AND PHOSPHOLIPID OXIDATION PRODUCTS(16)

The isolation of various classes or molecular species of phospholipids is essential for analysis of complex biological systems containing these important components. The preparative or analytical separation of phospholipids by adsorption chromatography on silica gel is, however, a tedious and time-consuming process. Furthermore, various molecular species of phospholipids may be separated only with difficulty by the use of established chromatographic techniques such as thin-layer and column chromatography.

In recent years, high-performance liquid chromatography (HPLC) has been used effectively for the analysis of several neutral lipids; more

recently this technique has also been applied to the separation of molecular classes and molecular species of phospholipids. Because of the difficulties inherent in the preparative or analytical separation of phospholipids by older chromatographic techniques, the use of HPLC in purification and analysis should prove to be a welcome addition to the techniques used in phospholipid research.

SEPARATION OF CLASSES OF PHOSPHOLIPIDS BY NORMAL PHASE HPLC

The separation of classes of phospholipids has been readily achieved by normal phase HPLC techniques. Jungalawala, et al.(7) successfully separated sphingomyelin and phosphatidylcholine (PC) from phosphatidylserine (PS) and phosphatidylethanolamine (PE). A 10 μM silica column was used, and the solvent system employed was acetonitrile-methanol-water (65:21:14, v/v/v). With this system, the PS and PE classes could not, however, be resolved. Products were detected by the use of an ultraviolet (UV) detector operating at 203 nm, and as little as 1 mmol of a phosphatidylcholine containing at least one double bond could be detected under these conditions. The detector response is directly related to the unsaturation in the lecithin, highly unsaturated species being easier to detect than the more saturated molecular species.

Geurts van Kessel, et al.(18) also used a 10 μM silica gel column but employed n-hexane-2-propanol-water as the solvent. With this solvent system, PE, phosphatidylinositol (PI), PS, and lysophosphatidylcholine (lysoPC) may be resolved, but sphingomyelin and PC are not separable. Sphingomyelin and PC are, however, separated with the acetonitrile-methanol-water system, and the use of 10 μM silica gel and the two solvent

systems (acetonitrile-methanol-water or n-hexane-2-propanol-water), effectively allows separation of PE, PI, PS, lysoPC, PC, and sphingomyelin. Geurts van Kessel, et al. also noted that it is even possible in some cases to achieve separation of molecular species within that class by the use of normal-phase HPLC on silica gel. This was a preliminary observation, however, and the separation of only two molecular species was reported.

Table 3.1

Elution order of phospholipid classes on μ-Silica and μ-Bondapak-NH_2[a]

Column	PA	PS	PC	PE	PI
Silica gel[b]	1	4	5	2	3
μ-Bondapak-NH_2[c]	1	2	3	4	5

[a]PA, phosphatidic acid; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol.

[b]Jungalawala, et al.(7); Geurts van Kessel(18).

[c]Kiuchi, et al.(19).

Kiuchi, et al.(19) separated phospholipid molecular classes with a 10 μM porous anion exchanger, μ-Bondapak-NH_2. A chloroform-methanol-water mixture was used as the eluent, and PS, PC, PE, and PI were resolved. As demonstrated in Table 3.1, the elution order of phospholipid molecular classes differs on this μ-Bondapak-NH_2 column from that observed on 10 μM silica.

Although methods exist for the separation and identification of lecithin molecular species, these methods (which include argentation chromatography and countercurrent distribution) are laborious and time-consuming and the resolution of some molecular species is difficult. Reverse-phase chromatography would appear, in theory, to offer greater potential for separation of molecular species than these techniques, since the deciding separation factor in reverse-phase chromatograhy is the lipophilic character of the lecithin acyl substituents, the fatty acids. These acyl substituents are precisely the component of lecithin that differs from one molecular species to another, and reverse-phase chromatograhy should, then, be the method of choice for separation of individual molecular species.

Arvidson(20) has reported that molecular species of egg yolk and rat liver lecithins may be separated on a nonionic partially alkylated Sephadex column. The resolution of lecithin molecular species with this reverse-phase open column separation was according to fatty-acid chain length and the degree of unsaturation of the fatty acids (acyl esters of the lecithin). As an example, these workers separate egg lecithin into four components (A-D). Component A, the first eluted fraction, contains primarily 16:0 and 18:2 with a smaller amount of 22:6 in the lecithin acyl chains. Fraction B consists of 16:0, 18:0; fraction C is primarily 18:0, 18:2; and fraction D is essentially 18:0, 18:1. Thus an equivalent carbon-atom number could be defined as the total number of acyl-chain carbon atoms minus the total number of double bonds, and the separation of

the molecular species roughly parallels this equivalent carbon-atom number. This pioneering work by Arvidson laid the groundwork for further experiments with reverse-phase HPLC and indicated the validity of the concept that lecithin molecular species could be separated on the basis of the lipophilic character of the acyl chain substituents. The disadvantages of this approach is the fact that the alkylated Sephadex is not readily available and must be prepared in two synthetic steps from Sephadex G-25. Further, gravity chromatograhy has disadvantages of inconvenience when compared to HPLC.

Table 3.2

Retention Volumes for Lecithin Molecular Species on Bondapak-C_{18}

Molecular species	R_1=14:0, R_2=14:0	16:0, 16:0	16:0, 18:2	16:0, 18:1	18:1, 18:1	16:0, 18:0	18:0, 16:0
Retention volume(ml)	18.3	34.7	29.1	35.5	35.5	49.6	49.6

The first HPLC separation of a variety of lecithins was carried out by Porter, et al.(21). Ten different synthetic lecithins were analyzed by reverse-phase HPLC. Both a 10 μM Bondapak-C18 and a 10 μM-Bondapak fatty acid analysis column (Waters Associates) were found to be useful for the separation of the lecithins. For example, with the 10 μM-Bondapak C_{18} column and methanol-water-chloroform (100/10/10, v/v/v) the retention volumes for the ten lecithin molecular species are as shown in Table 3.2. As can be seen from Table 3.2, the concept of an effective carbon number applies in this separation with the determining retention factor being the total number of acyl chain carbon atoms minus the number of double bonds present in the lecithin.

The selectivity of the C_{18} and fatty acid analysis columns are different and most of the molecular species that were not separable on the C_{18} column could be resolved on the fatty acid column. In fact, only two of the lecithins presented in Table 3.2 could not be separated by a combination of the two columns. The two unresolved lecithins contained the same acyl substituents, but the position of substitution was reversed on the glycerol chain (i.e., 1-palmitic, 2-stearic lecithin could not be separated from 1-stearic, 2-palmitic lecithin).

Commercial egg lecithin was separated into four compounds by reverse-phase HPLC on a C_{18} column. The HPLC separation of egg lecithin is strikingly similar to the reverse-phase alkylated Sephadex open column separation of Arvidson(20). The primary components of the four fractions (A-D) of the HPLC separation are as follows: A, 16:0, 18:2; B, 16:0, 18:1; C, 18:0, 18:2; and D, 18:0, 18:1.

Detection of the lecithins was achieved by UV absorption at 240 nm. The solvent used in this separation was methanol-water-chloroform (100/10/10, by volume), and it is impractical to use wavelengths lower than 240 nm because chloroform absorbs UV light. The chromatogram of lecithins is dramatically dependent on the previous exposure to oxygen of the material. The amount of polyunsaturated lecithins present in the mixture would be relatively depleted when significant exposure to oxygen had occurred prior to analysis.

More recently, improvements in the RP-HPLC separation of lecithins were reported. Crawford, et al.(14) separated soy PC into its major molecular species by RP-HPLC using an aqueous methanol gradient, that allowed detection of the various species by their absorbance at 206 nm.

Six fractions were collected and their fatty acid composition determined by GC. One fraction contained diLPC in 93% purity.

The aqueous methanol solvent system was modified by Compton, et al.(22). They found that small concentrations of strong mineral acids (such as 0.01 M H_3PO_4) in the eluent in addition to elevated column temperatures (60°C) were useful in improving the efficiency of the PC-separation.

The most effective separation of lecithins reported so far was recently published by Smith and Jungalawala(23). They described the separation of egg lecithin into 13 separate peaks, on a Nucleosil-5-C,8 reverse phase column with methanol-1 mM phosphate buffer pH 7.4, 95:5 (v/v) as the solvent. They suggest that critical lecithin pairs that are not resolved by this method can be separated by argentation HPLC.

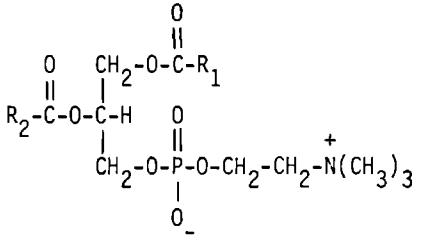
HPLC OF LECITHIN OXIDATION PRODUCTS

Since the oxidation of lecithins is potentially an important biological process, it seemed worthwhile to investigate in a bit more detail the oxidation of polyunsaturated lecithins. As noted earlier, we observed differences in egg lecithin molecular species after exposure to air, but the chemical fate of the oxidized lecithins was not established. Because of the complexity of egg lecithin mixtures, we decided to study the fate of lecithin oxidation in a synthetic lecithin, containing linoleic acid, since the oxidation and separation of oxidation products of this acid was previously well established (see Chapter 2). The oxidation of 1P,2L-PC and subsequent separation of the lecithin and the oxidized lecithin are described in this chapter (see also reference 24). During the course of

this study we became aware of similar work on soybean phospholipids being carried out by Crawford, et al.(14). Different lecithin species were separated and diL-PC was isolated from a soybean lecithin mixture. The air oxidation of diL-PC was carried out and the oxidized product isolated by RP-HPLC, using 95% MeOH 5% H_2O as the eluent.

3.2 RESULTS

3.2.1 LECITHIN SYNTHESIS

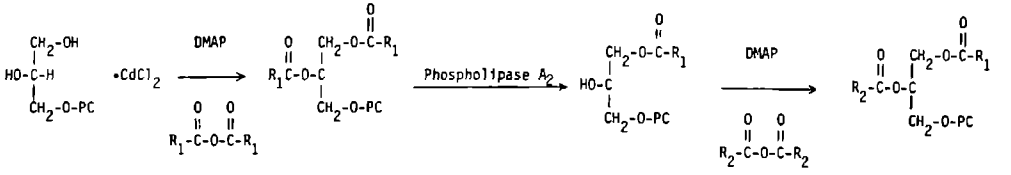


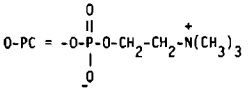
	R_1	R_2
1 diP-PC	16:0	16:0
2 diL-PC	18:2	18:2
3 1P,2L-PC	16:0	18:2
4 1S,2A-PC	18:0	20:4

Lecithins 1-4 were prepared by the method reported by Khorana, et al.(25). Thus Glycero Phosphoryl Choline-$CdCl_2$ complex was reacted with 4-8 equivalents fatty acid anhydride using 3-4 equivalents dimethylamino-

pyridine as an acylating agent. This was found to be a convenient one step synthesis to the symmetrical lecithins. The mixed lecithins were obtained by selectively hydrolyzing the fatty acid at the C2 position (R_2-COOH) using Phospholipase A-2 (snake venom, Crotalus Adamanteus or Naja Naja), and subsequently reacylating the C2 position with fatty acid anhydride and dimethylaminopyridine. The acylation takes place in 25-70% yield, phospholipase A-2 hydrolysis in 100% yield. The synthetic method described here can be represented by the scheme below:

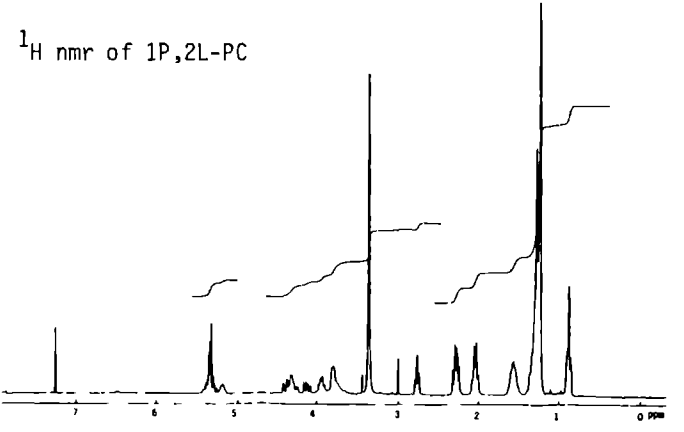
Scheme 3.5





These synthetic lecithins were characterized by ^{1}H and ^{13}C nmr(36), TLC and HPLC.

Figure 3.2 ^{1}H nmr of 1P,2L-PC



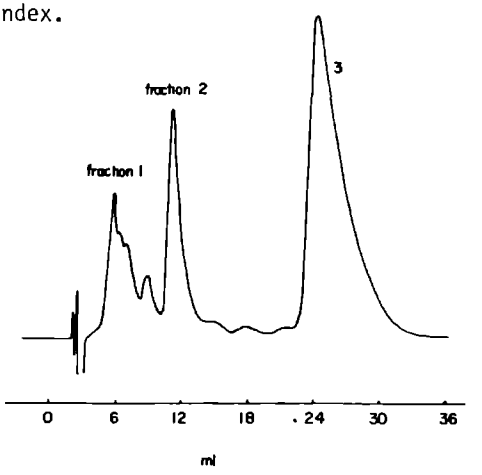
3.2.2 AUTOXIDATION OF 1P,2L-PC

1-Palmitic-2-linoleic-phosphatidylcholine reacts readily with oxygen when neat films, aqueous emulsions, or solutions (t-butanol, benzene solvent) of the lecithin are exposed to oxygen. The oxidation of thin films is particularly rapid at room temperature but dilute solutions are considerably more stable to air than the neat lecithin.

The reaction of the polyunsaturated lecithins with oxygen is inhibited by t-butylated hydroxy toluene (BHT), a known free radical inhibitor, and the oxidation is thus apparently a free radical chain process (see Discussion).

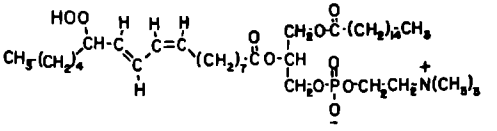
After exposure of neat 1-palmitic-2-linoleic-phosphatidylcholine to oxygen at 25°C for 16 hr, significant oxidation of the lecithin had occurred. Figure 3.5 shows the HPLC chromatogram obtained from such an oxidation, with two new fractions, 1 and 2, being observed in addition to the starting lecithin, 3. Oxidation of 275 mg of 1-palmitic-2-linoleic-phosphatidylcholine for 16 hr led to 32 mg fraction 2 and 6.6 mg fraction 1 as isolated by HPLC. By following an oxidation over time with HPLC, it was determined that fraction 2 was the first formed lecithin oxidation product. When fraction 2 was isolated, allowed to stand at room temperature and then re-chromatographed, significant amounts of fraction 1 had been formed from 2. Thus, fraction 1 appears to be a secondary oxidation product formed by decomposition of the compound(s) making up 2. Fraction 2 is apparently identical to the previously reported(24) contaminant of 1-palmitic-2-linoleic phosphatidylcholine formed upon storage at -20°C for several months.

Figure 3.3 HPLC chromatogram of product mixture obtained from a 16 hr oxidation of 1-palmitic-2-linoleic phosphatidylcholine. Solvent, CH_3OH-H_2O-$CHCl_3$ (100:10:10, v/v); 10 μ C-18 reverse phase column; detection by refractive index.



Fraction 2 showed a maximum in the ultraviolet at 230-235 nm, indicating the presence of a conjugated diene. Further, it was shown to be a peroxide by the potassiumiodide/starch test. NMR spectroscopy of fraction 2 showed that the bisallylic protons (2.8 δ) -CH=CH-CH_2-CH=CH- present at C-11 of linoleic acid had disappeared and the vinyl region had been broadened, an observation consistent with the formation of a conjugated diene. We thus tentatively concluded that fraction 2 was a diene hydroperoxide, or a mixture of various diene hydroperoxides, like 3a.

Triphenylphosphine reduction of3a produced a new product,3b, that was no longer peroxide positive, but still contained a conjugated diene



3a

as determined by its UV spectrum. Snake venom hydrolysis of 3b followed by HPLC analysis of the resulting free fatty acid led to a HPLC chromatogram which is very similar to Fig. 2.1. Four major products were observed in the chromatogram, and these four products have been assigned the structures 5a-5d. 5a, the first hydroxy fatty acid eluted, contains a 13-hydroxyl group with 9-cis,11-trans diene stereochemistry (see p. 34). 5b is a 13-hydroxy-9-trans,11-trans compound and 5c and 5d are the 9-hydroxy trans,cis and trans,trans dienes, respectively.

These structural assignments were based on 1) Comparison of the HPLC chromatogram of the products obtained after reduction and hydrolysis of the 1P,2L-PC autoxidation mixture with that of the hydroxylinoleates obtained from linoleic acid autoxidation. 2) Spectral comparison (ir and uv) of the products derived from the autoxidation described here with hydroxy linoleates obtained as described in Chapter 2. 3) GC-MS of the methyl esters after hydrogenation and silylation as described in Chapter 2. A more detailed study on the distribution of the product hydroperoxides is described in Chapter 4. 4) 1P,2L-PC emulsion autoxidation products were checked by TLC and GC to determine if any other major products besides hydroperoxides were formed in this reaction. Mead, et al.(13) have suggested that inter- and intramolecular additions leading to epoxides and epoxy alcohols may take place in nonenzymatic autoxidations in membranes. Studies on the autoxidation of linoleic acid monolayers revealed that epoxides were the major products formed from silica gel adsorbed monolayers. Hydroperoxides and epoxy alcohols were minor products under these circumstances. Thus we hydrolyzed a reduced 1P,2L-PC autoxidation mixture to the corresponding oxidized fatty acids by Phospholipase A-2 hydrolysis. Reduction was carried out with $P\emptyset_3$. It has been established

that neither $P\emptyset_3$(26) nor Phospholipase-A2(27) affects the formation of epoxy fatty acids in this reaction sequence. The oxygenated fatty acids obtained this way were analyzed by TLC, GC and GC-MS. The fatty acid mixture obtained from 1P,2L-PC autoxidation was compared with a mixture of linoleic acid epoxides, hydroperoxides and epoxy alcohols as otained from autoxidation of linoleic acid monolayers adsorbed on silica gel. TLC and GC could readily distinguish between the products formed from linoleic acid monolayer autoxidation. However when the chromatograms of both monolayer (on silica gel) and bilayer (aqueous KCl 50 mM) autoxidations were compared, it was concluded that only trace amounts of epoxides and epoxy alcohols were formed in bilayer autoxidations. Thus autoxidation of 1P,2L-PC aqueous emulsion unlike linoleic acid monolayers adsorbed on silica gel generates hydroperoxides as the major products. Autoxidations of 1P,2L-PC in a benzene/t-butanol solution and in aqueous 50 mM KCl emulsion were found to follow a very similar pattern. However, more t,t products seemed to be formed from the benzene/t-butanol solution autoxidation of 1P,2L-PC as compared to the neat and emulsion autoxidation.

3.2.3 AUTOXIDATION OF EGG LECITHIN

Egg lecithin contains two PUFA's, linoleic acid (17%) and arachidonic acid (4%), as reported by Ansell, et al.(28) (see Table 3.3). It has frequently been used as a model system in oxidation studies. To evaluate the possible use of this natural lecithin mixture for our studies, we carried out the autoxidation of an aqueous egg lecithin emulsion and followed the reaction by uv.

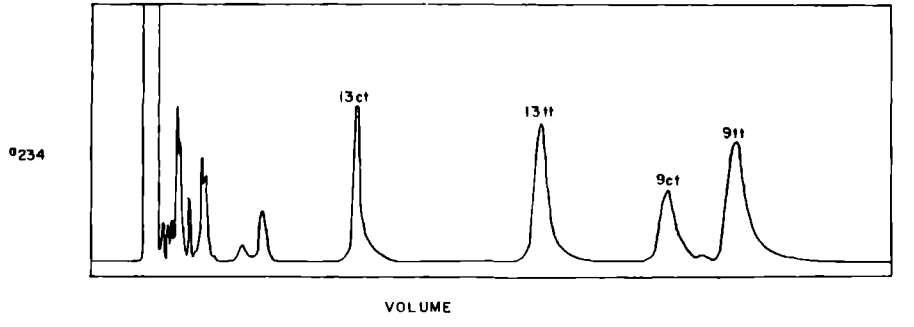
Table 3.3

Fatty acid composition of egg lecithin(28)

Fatty acid	mole %
16:0	32
18:0	16
18:1	30
18:2	17
20:4	4

Purified egg lecithin(29) was emulsified in 50 mM KCl and exposed to air. Autoxidation was relatively slow and it took 45 hr before the PUFA's present in egg lecithin showed 10% conjugation by UV (234 nm). The autoxidation mixture was reduced, hydrolyzed and analyzed by HPLC (see Fig. 3.6).

Figure 3.4 Chromatogram of reduced and hydrolyzed egg lecithin after autoxidation for 45 hr



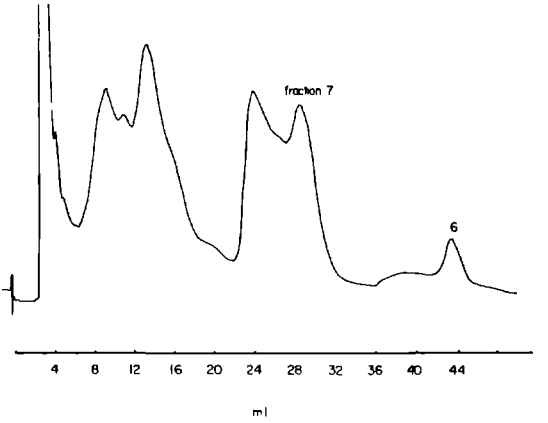
The chromatogram showed only linoleic acid hydroxides, no arachidonic acid alcohols were present in substantial amount. Thus egg lecithin can be used in studies of autoxidation of PUFA's, however, one has to be aware that the composition of egg lecithin is dramatically dependent on its history. We have reported earlier(16,21), that substantial oxidation had taken place in some of the commercial samples which we had analyzed by RP-HPLC. The amount of polyunsaturated lecithins present in a commercial sample would be relatively depleted when significant exposure to oxygen had occurred prior to use. Careful analysis of the fatty acids present in a commercial lecithin mixture is therefore necesary when studying lipid oxidations.

3.2.4 AUTOXIDATION OF 1S,2A-PC

1-Stearic-2-arachidonic-phosphatidylcholine, 4, is even more reactive towards oxidation than was the 1-palmitic-2-linoleic lecithin. After only 5 hr, significant oxidation of 4 had occurred and the HPLC trace shown in Figure 3.7 was obtained. As can be seen from the chromatogram, several products are formed from the lecithin. Separation of the various oxidation products by HPLC followed by: 1) triphenylphosphine reduction, b) snake venom hydrolysis, and c) HPLC analysis of the hydroxy arachidonate methyl esters(30) led to the conclusion that the fraction eluting from the HPLC column at 28 mL (fraction 7) contained the primary products of 4 oxidation, the arachidonic ester hydroperoxides. The product distribution of hydroxy arachidonate methyl esters obtained by the sequence outlined here was very similar to the distribution obtained from air oxidation of arachidonic acid methyl ester(30). The air oxidation of

1S,2A-PC thus follows a course similar to oxidation of 1-palmitic-2--linoleic-phosphatidylcholine. That is, conjugated diene hydroperoxides are formed first and these primary compounds decompose to give a complex mixture of products.

Figure 3.5 HPLC chromatogram of product mixture obtained from a 5 hr autoxidation of 1-stearic-2-arachidonic phosphatidylcholine. Solvent, CH_3OH-H_2O-$CHCl_3$ (100:12:10, v/v); 10 μ C-18 reverse-phase column; detection by UV at 240 nm.



3.3 DISCUSSION

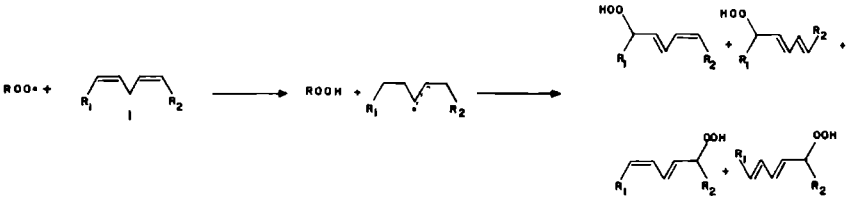
Polyunsaturated fatty acids and esters are particularly prone to undergo air oxidation by a free radical chain mechanism. Autoxidation has been investigated in detail(31) and the generally accepted mechanism involves the two propagation steps (I) and (II). The overall rate of autoxidation is related directly to kp, the rate constant of hydrogen atom

$$R\text{-}O\text{-}O\bullet + R'\text{-}H \xrightarrow{kp} R\text{-}OOH + R'\bullet \qquad (I)$$

$$R'\bullet + O_2 \longrightarrow R'\text{-}OO\bullet \qquad (II)$$

abstraction by a peroxy radical. This rate constant, kp, is dramatically dependent on the structure of R'-H and the homoconjugated diene present in linoleic as well as other polyunsaturated fatty acids and esters is a particularly good hydrogen atom donor(32). Abstraction of the bisallylic hydrogen of the homoconjugated diene leads to a free radical that is stabilized by delocalization over five carbon atoms and reaction of this radical with molecular oxygen followed by hydrogen atom abstraction leads to the hydroperoxides shown (Scheme 3.6):

Scheme 3.6



Polyunsaturated lecithins are noted for their instability in air. 1-Palmitic-2-linoleic-phosphatidylcholine, 3, readily undergoes air oxidation and our evidence indicates that a mixture of four hydroperoxides is formed. Reduction of the hydroperoxides with triphenylphosphine to give the alcohols is followed by snake venom hydrolysis, a method known to specifically remove the fatty acid ester from position 2 of the glycerol moiety. The four hydroxy fatty acids formed by this sequence are shown here to be identical to the alcohols formed from autoxidation of free linoleic acid or its methyl ester(33).

The autoxidation of polyunsaturated lecithins thus appears to follow a course similar to the autoxidation of the free fatty acids or esters, with conjugated diene hydroperoxides being the primary products formed in the free radical chain process. Both the hydroperoxides and the alcohols are not separated from the starting material by normal phase chromatograhy, whereas RP-HPLC or RP-TLC readily separate these compounds from the starting lecithin.

The procedures reported here make available lecithin hydroperoxides for potential biological study. Further, the methods outlined in this chapter provide a framework for the study of the oxidation of more comple> mixtures of lecithins such as those found in biological membranes. It must be emphasized that it is necessary to use absolutely fresh lecithin preparations when studying biological mixtures. Polyunsaturated fatty acids and lecithins oxidize faster with increasing unsaturation. Thus polyunsaturated lecithins with more than two double bonds could be completely depleted when significant exposure to oxygen had occurred prior to use. It seems more practical to use synthetic lecithin preparations whether in oxidations of single lecithins or mixtures of lecithins (and

other lipids). However, natural sources of lipids can be used when extreme care is taken to prevent oxidation.

3.4 EXPERIMENTAL

Fatty acids were obtained from NuChekPrep (Elysian, MN) and used without further purification. Glycerol Phosphoryl Choline-$CdCl_2$ (GPC-$CdCl_2$) complex and lysolecithins were obtained from Serdary Labs (Canada). Phospholipase A-2 (snake venom, Crotalus Adamanteus or Naja Naja) and egg lecithin (type III-E) were obtained from Sigma Biochemical Co. (St. Louis, MO).

LECITHIN SYNTHESIS

Symmetrical lecithins were synthesized from commercially available GPC-$CdCl_2$ and mixed lecithins from lysolecithins by the method reported by Khorana, et al.(25). Khorana used 2.5 mole anhydride and 2.0 mole dimethyl aminopyridine for every one mole of GPC-$CdCl_2$. We found that a larger excess of anhydride and base resulted in higher yields and faster reactions. If the anhydride is not precious, 6 to 8 mole/mole GPC-$CdCl_2$ can be used with good results, along with 3 to 4 mole of base per mole of GPC-$CdCl_2$. Lysolecithins were prepared from lecithins using snake venom hydrolysis. Extreme care was taken to prevent oxidation when PUFA anhydrides were used for acylation. Thus the reaction time was shortened to 24 hr and argon was flushed through the system, keeping oxidative degradation to a minimum. Under these conditions, however, oxidation was

still substantial and HPLC purification necessary. Yields of the crude product were 25-70%. The products were characterized by ^{1}H and ^{13}C nmr and were pure by TLC and HPLC.

AUTOXIDATION OF NEAT LECITHINS

A methanol solution of a lecithin was evaporated to dryness under vacuum so that the lecithin formed a thin layer on the inside of a round bottom flask. The flask was then exposed to a stream of dry air and the reaction was monitored by UV, RP-TLC and RP-HPLC. After about 10% conversion, the primary product was isolated by RP-HPLC and analyzed.

AUTOXIDATION OF LECITHIN SOLUTIONS

A methanol solution of a lecithin was evaporated to dryness under vacuum and the lecithin redissolved in a mixture of benzene and t-butanol (9:1) or benzene. The oxidation was carried out in a 10 mL two neck flask with a condenser (cooling fluid temperature was approximately 0°C) in connection with open air via a drying tube ($CaCl_2$) to provide a constant oxygen supply. The solution was stirred magnetically and kept at a constant temperature with an oil bath and thermoregulator. The reaction was followed by UV, RP-TLC and RP-HPLC and after approximately 10% conversion the solvent was removed under vacuum and the product analyzed.

AUTOXIDATION OF LECITHIN EMULSIONS

A methanol solution of a lecithin was evaporated to dryness under vacuum and the lecithin emulsified in 50 mM KCl as described by Van Deenen, et al(34). The autoxidation procedure for emulsions was analogous to that for solutions.

TLC

Normal phase TLC was carried out on silica gel 60F-254 precoated plates (Merck), using $CHCl_3$-MeOH-H_2O (70:25:4, v/v) as the eluent. This

gives a good separation of lecithin from lysolecithin(25), but an incomplete resolution of 1P,2L-PC and its primary oxidation product. The oxidized product moved somewhat slower than the starting material.

Reverse phase TLC (RP-TLC), on precoated silanized silica gel 60F-254 (Merck) was carried out using MeOH-NH_4OH 0.6%-$CHCl_3$ (7:2:1, v/v) as the mobile phase. This method gave an excellent separation of the lecithin and its primary oxidation products, and was therefore useful in monitoring the oxidation in an early stage. However, in a later stage, other oxidation products gave rise to very large spots interfering with both the starting material and the primary oxidation products. Phospholipids were visualized with molybdenum blue spray (zinzadze reagent)(25) and peroxides were detected with the KI/starch assay(35). Reverse phase TLC could also be used to separate different lecithins. Thus complete resolution of di-Myristic-PC, di-Palmitic-PC and di-Stearic-PC was obtained using RP-TLC 10 cm plates, with the same solvent as used for the separation of lecithins and their oxidation products (vide supra). The R_f values for the three lecithins mentioned were respectively, .38, .28 and .15.

HPLC

A μ Bondapak C-18 column (Waters Associates, Milford MA) was used with CH_3OH-H_2O-$CHCl_3$ (solvent proportions described in figure captions) as the eluent (14). The flow rate was 3.0 mL/min; detection was by refractive index or UV (240 nm). For preparative purposes, large amounts (20 mg in 200 μL solvent) of the oxidation mixture could be separated in one injection.

Hydroxy fatty acid methyl esters were separated by normal phase HPLC using a Waters Associates 10 μ Porasil column (7.8 mm ID x 30 cm) and .5% isopropanol in hexane as the eluent. The flow rate was 4.0 mL/min,

detection was by UV at 235 nm. Free hydroxy fatty acids were analyzed using acetic acid-isopropanol-hexane (1:16:983, v/v) as solvent and the same 10 μ column as was used for the hydroxy fatty acid methyl ester analysis. The normal phase separations were significantly improved when a 5 μ Whatman Partisil analytical column was used at flow rate 1.5-2.0 mL/min. The solvents used were essentially similar to that measured for the 10 μ semi-preparative column (vide supra).

ANALYSIS OF LECITHIN AUTOXIDATION PRODUCTS

Fractions collected by reverse-phase HPLC were concentrated in vacuo at ambient temperature until only water remained. The residue was freeze dried or evaporated to dryness after adding CH_2Cl_2 and MeOH, to obtain azeotropic water removal. Samples of the isolated product were reduced with triphenyl phosphine (see Chapter 2). The reduced lecithin was then specifically hydrolyzed at the C_2 position with phospholipase A_2 (Sigma, Crotalus adamanteus or Naja Naja snake venom) to isolate the 18:2 hydroxy fatty acids(25). This was done at ambient temperature to keep decomposition or isomerization of the product to a minimum.

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CHAPTER 4

THE COOXIDATION OF LINOLEIC LECITHINS WITH A SATURATED LECITHIN, STEROIDS, LINOLEIC ACID AND α-TOCOPHEROL

FACTORS CONTROLLING THE STEREOCHEMISTRY OF PRODUCT HYDROPEROXIDES

4.1 INTRODUCTION

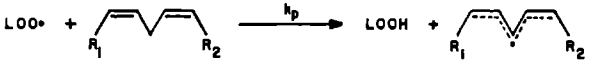
4.1.1 MECHANISM OF LINOLEIC ACID BULK PHASE AUTOXIDATION

Studies of the autoxidation of polyunsaturated fatty acids have been fragmentary and incomplete. While enzymatic oxidations have received considerable attention(1-3), random autoxidation, which appears to be an important process in vivo as well(4), has only recently been studied in a systematic way. Product mixtures obtained in polyunsaturated fatty acid random autoxidation are complex and the primary processes leading to products have not been firmly established. It is only since the development of sophisticated separation techniques such as HPLC(5,6) that systematic product studies have been within the realm of possibility. The primary products of lipid autoxidation, hydroperoxides, can be analyzed by HPLC, and after reduction to the corresponding alcohols, they are all separable by this technique. Both Chan(7-10) and Porter(11-16) have extensively used this method to study lipid hydroperoxides and the autoxidation process leading to these hydroperoxides. It was observed in the course of our autoxidation studies, that the concentration of linoleic acid affects the product distribution of the product hydroperoxides. This led to a systematic survey resulting in a general mechanism for PUFA autoxidations, as outlined in Scheme 4.7. This and other kinetic and

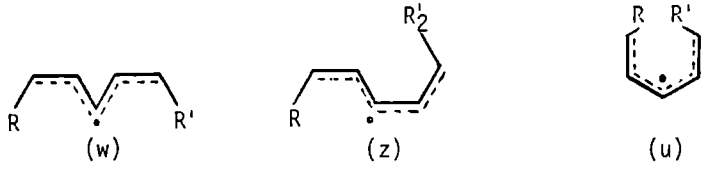
product studies addressing important mechanistic questions in free radical lipid oxidation can be summarized as follows:

1. The hydrogens that are abstracted in a free radical oxidation of PUFA's are those that are attached to the bis-allylic carbons. The resulting pentadienyl radical is resonance stabilized and therefore other H's are not abstracted to any significant extent. The rate of H abstraction per bisallylic H (k_p/H) is 31 $mole^{-1}$ s^{-1} (methyllinoleate) at 30°C, allylic H's on the other hand are abstracted much slower, k_p/H being only 0.22 for methyl oleate(17). The first propagation step of linoleate autoxidation can therefore be represented as follows:

Scheme 4.1



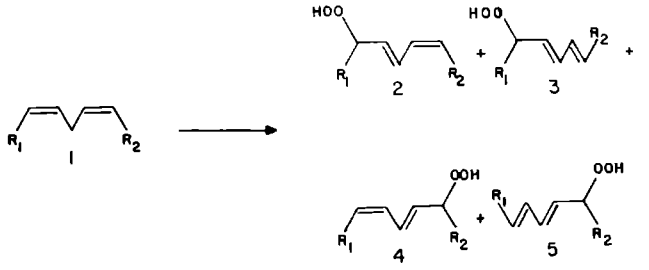
2. The conformation of a pentadienyl radical at temperatures above -60°C has solely the w structure(18,19). ESR studies on pentadienyl radicals in pentane showed that below -60° both the (w) and (z) conformations can be detected, above -60° only the less sterically hindered (w) conformation exists.



The sterically most hindered (u) configuration exists only in ring systems, such as a cyclohexadiene radical.

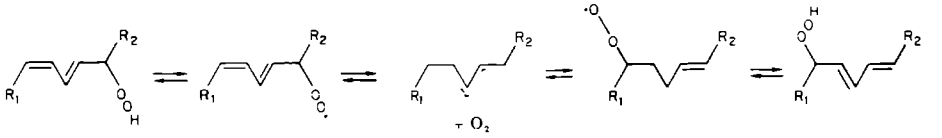
3. Autoxidation of linoleic acid, neat or in solution produces only four major compounds out of a possible total of eight isomers having the hydroperoxide group at positions 9 and 13. The cis-cis isomers and perhaps more significantly, the trans-cis isomers with the cis double bond adjacent to the hydroperoxide group are not formed to any appreciable extent(7).

Scheme 4.2



4. Thermal isomerization of methyl linoleate hydroperoxides takes place via β elimination of O_2 from a peroxy radical. Heating of the 13-methyllinoleate hydroperoxide in hexane at 40°C for 16 hr, resulted in >90% recovery of all four hydroperoxides 2-5(9). Using $^{32}O_2$ labelled methyllinoleate hydroperoxide it was found that atmospheric $^{36}O_2$ was incorporated in 2-5, during isomerization. $^{34}O_2$ incorporation was not detected in any appreciable amount. Thus oxygen addition to pentadienyl radicals is reversible.

Scheme 4.3



5. An extensive study of linoleic acid autoxidation under conditions of varying temperature and concentration was carried out in our laboratories(14). Preliminary observations relating to temperature(20) and concentration effects in linoleate oxidations were investigated in greater detail. Thus, linoleic acid was oxidized neat or in benzene solution at 10°, 30°, 37° and 50°C. The oxidation was carried out under air and was initiated by di-tert-butylhyponitrite. The extent of oxidation was measured by UV spectroscopy, the diene products having absorption maxima in the 230-235 nm region(5,7). Products of oxidation were analyzed by HPLC after reduction of the hydroperoxides with triphenylphosphine. A more detailed description of the analysis is given in Chapter 2. The following observations were made:

a. The total amount of 13 tc and tt isomers was in all cases very close to the total of the 9 tc and tt isomers.

b. The 13 tc/13 tt ratio was in all cases virtually identical to the 9 tc/tt ratio.

c. The (13 tc + 9 tc)/(13 tt + 9 tt) ratio was dramatically dependent on temperature and linoleic acid concentration. The total amount of trans cis products/total amount of trans trans products was plotted versus the linoleic acid concentration at various temperatures.

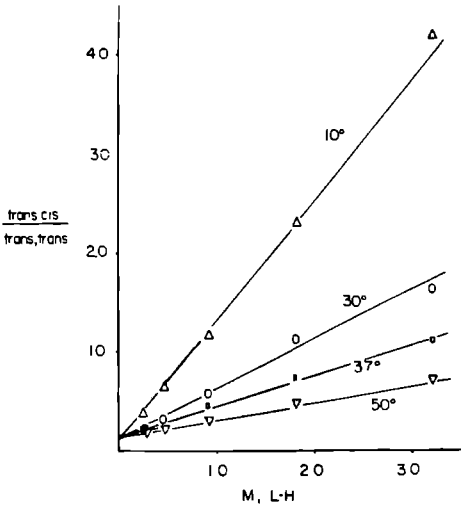


Fig. 4.1 Product ratio of trans,cis/trans,trans hydroxy fatty acids formed in autoxidation of linoleic acid (L-H) in benzene solution.

d. The product distribution was shown to be independent of the extent of oxidation, within the 2% total oxidation limit of this study. As an example, the product distribution of oxidation of 1.8 M linoleic acid in benzene at 30°C was identical when monitored at 0.5, 1, 2, 4, and 8 hr.

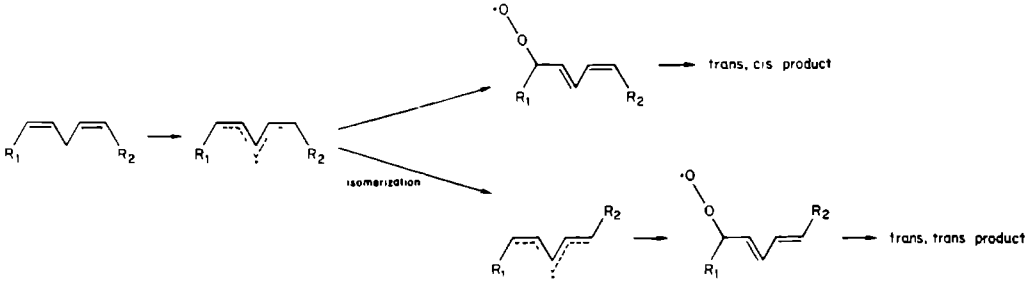
e. The product distribution was also independent of O_2 pressure: oxidations carried out under 1000 mm Hg or 80 mm Hg O_2 pressure gave identical results.

Thus the distribution of products derived from linoleic acid autoxidation is dramatically dependent on the concentration of linoleic acid and the temperature.

Any consideration of a mechanism consistent with these data must be concerned with the fact that the product distribution observed does not

change significantly during the time period (< 2% conversion) of oxidation. This observation suggests that the product ratios reported here are kinetically controlled and do not represent any significant isomerization of hydroperoxides once they are formed. This is a particularly important point since it has been recently shown(10) that linoleate hydroperoxides interconvert thermally (Scheme 4.3). This thermal isomerization has been shown to occur by β-scission of peroxy radicals formed from the hydroperoxides and the equilibration of hydroperoxides 2-5 has been demonstrated after thermolysis at 60°C for 48 hr. The shorter reaction times and lower temperatures apparently allow no significant isomerization of the product hydroperoxides, in the autoxidation study described here.

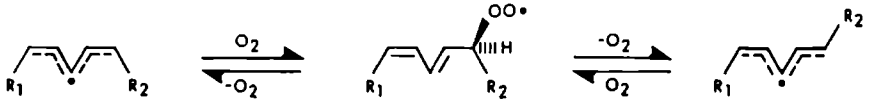
Scheme 4.4



A second mechanistic consideration is the lack of dependence of the product composition on oxygen concentration. It has been shown that the rate of autoxidation of linoleate is independent of oxygen pressure (between 100 and 760 mmHg)(17). These observations suggest that the chain-carrying radicals are primarily peroxy and not carbon radicals and

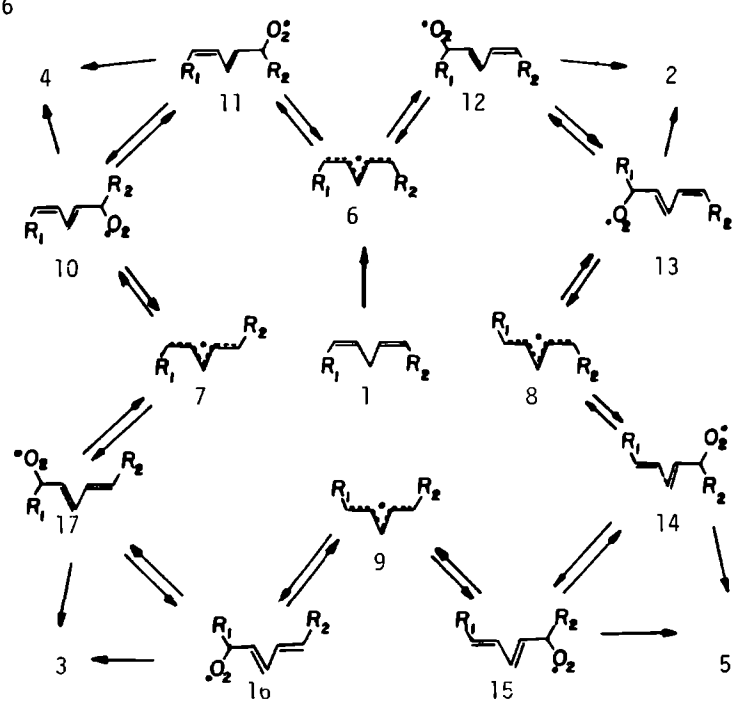
that isomerization of carbon radicals as shown in Scheme 4.4 is not a tenable mechanism. Further, it is difficult to account for the dependence of the trans,cis/trans,trans product ratio on fatty acid concentration if this carbon radical isomerization scheme is used as a format for discussion. However, the interconversion of a c,c pentadienyl radical and a t,c pentadienyl radical via O_2 addition and β elimination are in agreement with all observations:

Scheme 4.5



Based upon Scheme 4.5, the mechanism presented graphically in Scheme 4.6 was developed to account for the formation of products 2-5 from linoleic acid. It may also be used to explain the observed temperature and fatty acid concentration dependence of the trans,cis/trans,trans product ratios. In this mechanism, H-atom abstraction from fatty acid 1 leads to the W radical 6. Substitution about the Δ1 and the Δ4 partial double bonds of this substituted pentadienyl radical 6 must initially be cis since these bonds derive from the cis fixed fatty acid precursor.

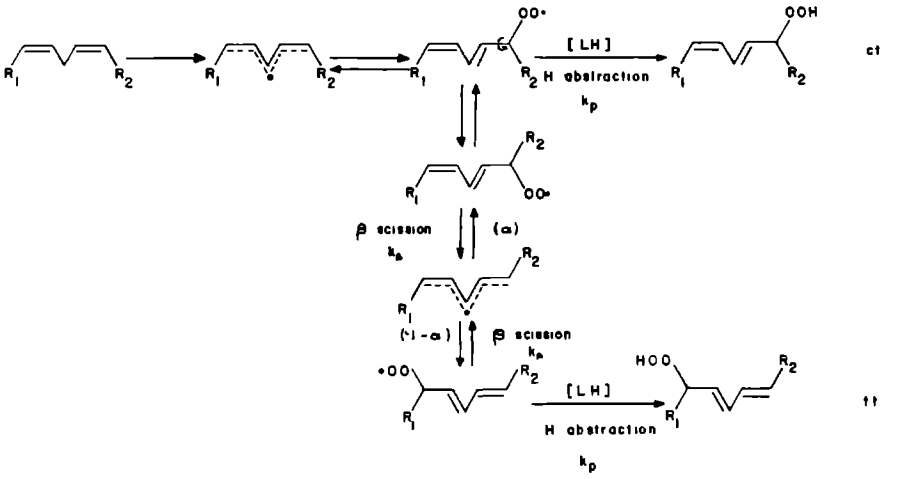
Scheme 4.6



Entrapment of 6 by oxygen gives peroxy radicals 10-13. Note that the pairs 10/11 and 12/13 are merely conformers. These conformers are independently represented, however, since scission of 11 yields the carbon radical 6, whereas scission from radical conformer 10 gives the new carbon radical 7. Competing with scission of the radical 10/11 is H-atom abstraction from fatty acid leading to the product hydroperoxide, 4. The crucial competition, then, that determines the trans,cis/trans,trans product ratio is (1) β-scission of peroxy radical 10/11 which ultimately

leads to trans,trans product or, (2) H-atom abstraction by 10/11 giving the trans,cis product. A simplified kinetic analysis based on Scheme 4.6 and radicals 11, 10, 7, and 6 is shown below:

Scheme 4.7



(A similar scheme based on the symmetry-related radicals 13, 12, 8, and 6 may also, of course, be developed.) Only the important product-determining steps are included in this analysis and the terms α and $1-\alpha$ are factors that represent the distribution of carbon radical 7 between the trans,cis peroxy radical (10/11) and trans,trans peroxy radical (16/17). In short, α and $1-\alpha$ are inserted to account for equilibration of peroxy radicals subsequent to the initial β-scission of 10/11. Steady-state assumptions lead to the equation:

$$\frac{\text{trans,cis}}{\text{trans,trans}} = \frac{k_p[LH]}{k_\beta(1-\alpha)} + \frac{\alpha}{(1-\alpha)} \qquad \text{(eq. 4.1)}$$

Equation 4.1 predicts a trans,cis/trans,trans product dependence that is directly related to L-H with the slope = $k_p/k_\beta(1-\alpha)$ and intercept = $\alpha/(1-\alpha)$. Analysis of the data presented in Figure 4.1 leads to α = 0.13 and k_p/k_β (10°C) = 1.1; (30°C) = 0.42; (37°C) = 0.27; (50°C) = 0.16 M^{-1}.

The value of k_p for linoleic acid autoxidation at 30 C is known to be 62 $M^{-1}s^{-1}$(17) and the k_β value may then be calculated and is found to be 144 s^{-1} (30°C). A plot of log (k_p/k_β) vs. 1/T gives a good linear correlation (r = 0.99) and from this the values $\Delta H_\beta^{\neq} - \Delta H_p^{\neq}$ = 10.5 kcal/mol and $\Delta S_\beta^{\neq} - \Delta S_p^{\neq}$ = 35 eu are found. Although the activation enthalpy of linoleate autoxidation propagation has not been reported, $\Delta H_p^{\neq}$ for most comparable autoxidation(21) falls between 6 and 8 cal/mol, giving an approximate $\Delta H_\beta^{\neq}$ of 16-18 kcal/mol.

6. The mechanism outlined above was further confirmed by another product study carried out in our lab by J. A. Khan(14). Linoleic acid (0.4 M) in benzene was cooxidized at 30°C with p-methoxyphenol, an excellent autoxidation inhibitor, present. Autoxidation did occur when p-methoxyphenol was added, albeit at a slower rate than with no added inhibitor. Products of autoxidations carried out in the presence of inhibitor were worked up in the normal way but an oxidation product of

p-methoxyphenol complicates the LC analysis of 2-5. In these studies, reproducible analysis of only 2 and 3, the trans,cis and trans,trans 9-substituted isomers, could be obtained. In Figure 4.2 is presented the product distribution of compounds 2 and 3 formed in oxidation as a function of added p-methoxyphenol.

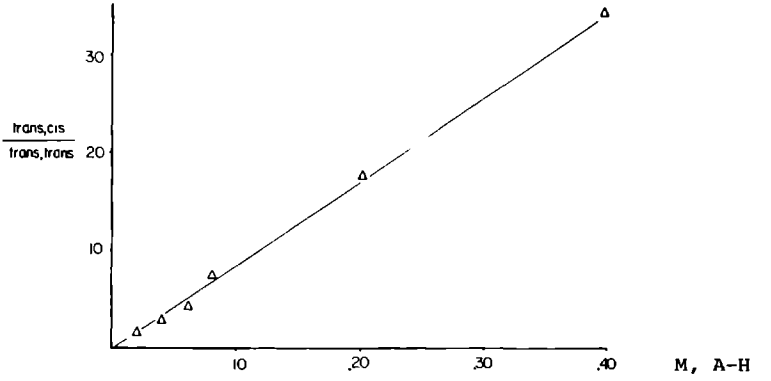


Figure 4.2 Product ratio of trans,cis/trans,trans hydroxy fatty acids formed in autoxidation of linoleic acid with added p-methoxyphenol (A-H).

If the competition between β-scission and H-atom abstraction of peroxy radicals determines the trans,cis/trans,trans product ratio, then reagents other than L-H that serve as H-atom donors may be added to the reaction mixture and alter the observed trans,cis/trans,trans ratio. This effect is observed with added p-methoxyphenol (A-H), an excellent H atom transfer agent(21). Thus, addition of A-H leads to an increased trans,cis/trans,trans ratio with as little as 0.25 M p-methoxyphenol increasing the product ratio from 0.23 (no added phenol) to over 25. In Figure 4.2 is presented a plot of the product ratio as a function of added A-H. The slope of this plot corresponds to $k_{inh}/k_{\beta}(1-\alpha)$ and, with k_{β} and α

known from autoxidation of the free fatty acid alone, k_{inh}, the rate constant for H-atom transfer from A-H to the peroxy radical, may be calculated.

Scheme 4.8

$$\text{L-OO}\bullet + \text{A-H} \xrightarrow{k_{inh}} \text{L-OOH} + \text{A}\bullet$$

The value of k_{inh} obtained in this way, 8.5×10^3 M^{-1} s^{-1} (30°C), is over two orders of magnitude greater than k_p, the rate constant for H atom transfer from L-H to the peroxy radical, LOO•. Cooxidation of L-H with other substrates in a manner similar to that described here for A-H offers an alternate competition method for determining rates of inhibition of autoxidation. Any added reagent should affect the trans,cis/trans,trans product ratio of L-H autoxidation and this ratio may then be used as a measure of the overall H atom donating ability of solvent and solutes present in the autoxidation.

$$KP = \sum_{i=1}^{n} k_{pi} [R_i\text{-H}] \qquad \text{(eq 4.2)}$$

For convenience this parameter is referred to as KP, and can replace kp[LH] in eq 4.1, to give a more general equation:

$$\frac{\text{trans,cis}}{\text{trans,trans}} = \frac{KP}{k_{\beta}(1-\alpha)} + \frac{\alpha}{(1-\alpha)} \qquad \text{(eq. 4.3)}$$

4.1.2 KINETIC STUDIES OF LECITHIN OXIDATIONS

1. The autoxidation of egg lecithin in hexane, benzene, ethanol and water was studied by Schreiber, measuring UV absorption and hydroperoxide formation(23). The rate of egg lecithin oxidation decreased in the order hexane, benzene, aqueous NaCl, and ethanol (96%). No oxidation took place in the latter solvent over a period of 70 hr.

A comparative study of the autoxidation of emulsions of Phosphatidylethanolamine (PE) and Phosphatidylcholine extracted from egg and soybean lipids was carried out by Corliss and Dugan(24). They measured extent of oxidation by oxygen uptake, diene and triene formation (uv), malonaldehyde formation (TBA-test) and the disappearance of two PUFA's (linoleic and arachidonic acid). They found that after the onset of initiation the rate of oxygen uptake was essentially the same for all lipid mixtures studied. They suggest that since these rates were measured during the straightline portion of the uptake curve, the oxidizabilities of lipid mixtures were the same for all four systems. Of the two major polyunsaturated fatty acids in egg phospholipids, arachidonic acid disappeared at a more rapid rate during autoxidation, while the concentration of linoleic acid decreased to a level that was relatively

constant. The induction period for phosphatidyl ethanolamine (PE) autoxidation was shorter than for phosphatidylcholine (PC), which could only be partially explained by the higher unsaturation of the PE's as compared to the PC's. The authors suggest that the nitrogen moiety could also be responsible for the difference in induction periods for PE and PC.

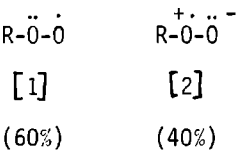
2. A kinetic study on the autoxidation of lecithins was carried out by Barclay and Ingold(25) almost simultaneous to our products study. They reported on the autoxidation of egg lecithin in aqueous NaCl (0.1 M) and in chlorobenzene. The autoxidation was initiated by di tert-butyl hyponitrite (DBHN) which completely dissolved in the bilayer (microscope studies). The fraction of the initiator escaping from the solvent cage, a, was found to be 0.66 ± 0.06 in chlorobenzene, and 0.043 ± 0.008 in aqueous NaCl. The oxygen uptake in chlorobenzene was directly proportional to [egg lecithin] x $[In]^{1/2}$ and virtually independent of O_2 pressure. In aqueous dispersion the egg lecithin oxidation was proportional to $[In]^{1/2}$ and almost independent of O_2 pressure. The rate of oxygen uptake was independent of the concentration of lecithin dispersed in the aqueous phase, since the effective egg lecithin concentration in a bilayer is the same as that of neat egg lecithin. Thus, it was concluded that the classical rate law

$$\frac{-d[O_2]}{dt} = \frac{k_p[RH]R_i^{1/2}}{(2k_t)^{1/2}} \qquad \text{(eq. 2.1)}$$

is obeyed, for the autoxidation of egg lecithin in solution as well as in aqueous dispersion. However the oxidizability, $k_p/(2k_t)^{1/2}$, of egg

lecithin was very different in the two systems. In chlorobenzene $k_p/(2k_t)^{1/2}$ = 0.61 ± 0.09 $M^{-1/2}$ $s^{-1/2}$ and in 0.1 M aqueous NaCl the value of $k_p/(2k_t)^{1/2}$ which is obtained on the assumption that the egg lecithin concentration in the bilayer is 1.0 M is 1.65 ± 0.25 x 10^{-2} $M^{-1/2}$ $s^{-1/2}$ at 30°C. The authors suggest that the reduced oxidizability of egg lecithin in aqueous dispersion is due to a dramatic reduction of k_p, since k_t for 2° alkyl peroxyls in homogeneous solution (∿ 107 M^{-1} s^{-1} at 30°C) is generally close to the difussion controlled limit. It is suggested that the reason for this reduction in k_p lies in the polarity of the peroxy radical. The authors refer to the fact that the stabilizing canonical structure [2] makes a ∿ 40% contribution to the peroxy radical.

Scheme 4.9



[1] (60%) [2] (40%)

They estimate the dipole moment of the peroxy moiety to be ∿ 2.6 D and hypothesize that the peroxyl portion of the radical will rapidly diffuse out of the nonpolar autoxidizable lipophilic region in which it was formed, into the polar nonautoxidizable surface region of the bilayer. Chain propagation will therefore be retarded while the resulting increased local concentration of peroxyls near the surface will increase chain termination. That is, the nonhomogeneous distribution of peroxyls in the bilayer will lead to an apparent decrease in k_p and increase in 2 k_t. It was also suggested that this is consistent with the molecular structure of a very

efficient natural antioxidant, α-tocopherol, which seems to have its active site located in the polar phenolic OH group. The presence of the active site of the chain breaking antioxidant tocopherol in the hydrophilic region of the bilayer would make it an extremely efficient antioxidant if its target species, the peroxy radicals, are present in that same region as well. The consequences of this hypothesis for our product studies are discussed in the discussion part of this chapter, and in Chapter 5. Important kinetic parameters resulting from Barclay and Ingold's paper can be summarized as follows:

Table 4.1

Kinetic Parameters Reported by Ingold and Barclay(25)

	$k_p/(2k_t)^{1/2}$ $(M^{-1/2}\ s^{-1/2})$	k_p $(M^{-1}\ s^{-1})$	k_t $(M^{-1}\ s^{-1})$	a)'	LH (M)
egg lecithin in ØCl	0.61	-	-	.66	.0025-.0125
egg lecithin in aq. emulsion	0.0165	-	-	.043	1.0
methyl linoleate in ØCl	0.021	62	4.4×10^6	-	.1

)' cage escape efficiency, i.e., the ratio of radicals escaping the solvent cage over 2 x the total amount of initiator decomposed.

A study very much related to the initiation studies in the paper mentioned above, was reported by Winterle and Mill(26). They describe kinetic experiments with another lipophilic radical source, azobis[(2-n-butylcarboxy)propane], $[Me_2C(CO_2Bu)]N_2$, (ABCP) and a phenolic

antioxidant 2,6 di-tert-butyl-4-methyl phenol (BHT), both in aqueous emulsion and homogeneous solution at 50 C. Liposomal suspensions produced escape efficiencies (a) significantly smaller than corresponding model solvents of the same chain length. For example in dimyristic-PC bilayers cage escape fraction a was 0.25, while in methyl myristate a = 0.29. Similarly a in dilaurate-PC was 0.21, in methyl laurate a = 0.39. We note that the difference in a in emulsion(aq. PC) and solution (n-hexane) is small [a(hexane)/a(diMPC) $\sim$ 2.3] in comparison with [a(ØCl)/a(eggPC) $\sim$ 15] as reported by Ingold and Barclay for DBHN.

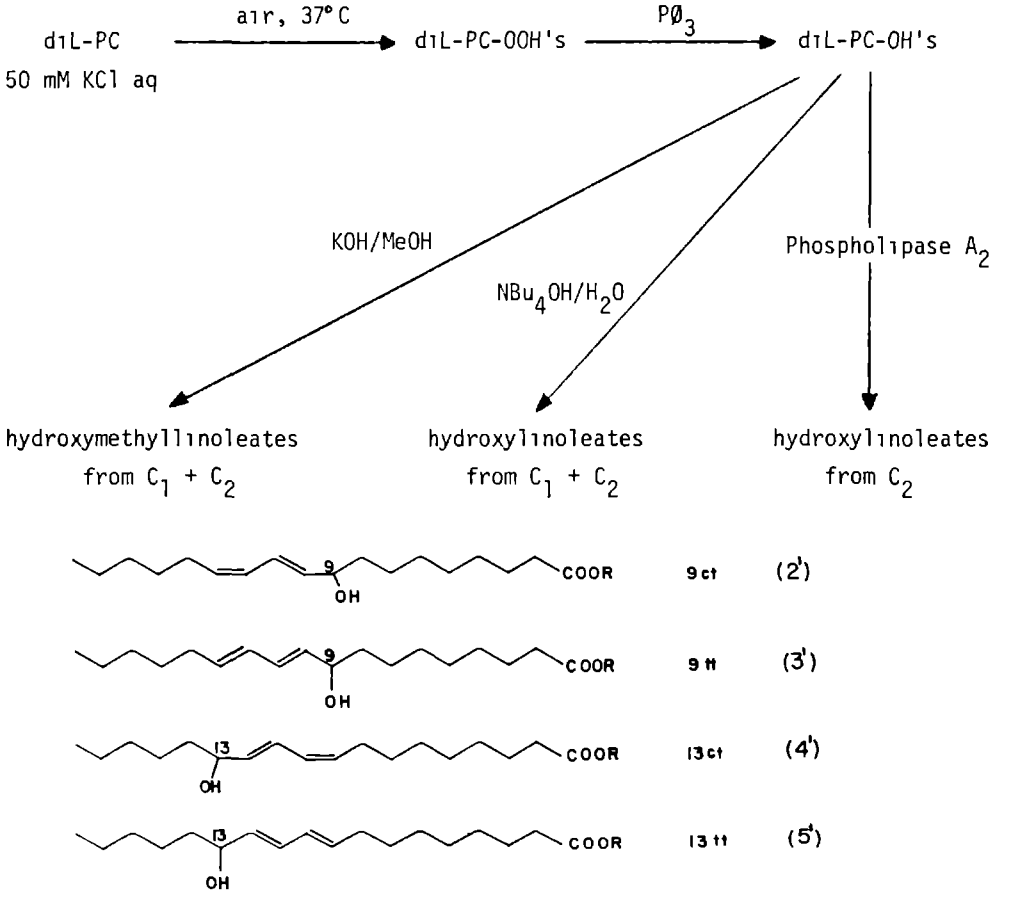
4.2 RESULTS

4.2.1 AUTOXIDATIONS OF 1P,2L-PC AND diL-PC IN THE PRESENCE OF DIP-PC, CHOLESTEROL AND 7-DEHYDROCHOLESTEROL

1P,2L-PC [18] and diL-PC [19] were autoxidized neat or in aqueous emulsion at 37°C(14). The dilinoleic lecithin was oxidized in mixtures with varying proportions of diP-PC [20]. Products of the oxidation were reduced with triphenylphosphine, and the esters were then hydrolyzed with Phospholipase A_2 affording the hydroxy fatty acids 2'-5', which were analyzed by HPLC. The oxidized phospholipid could also be hydrolyzed by tetrabutylammonium hydroxide to give 2'-5'. Transmethylation of the phospholipid with KOH/MeOH alternatively afforded the methyl esters of 2'-5' that could also be analyzed by HPLC. Essentially no difference in product

distribution was observed in reactions worked up by the different methods described here. The procedure, for example, for diLPC oxidation analysis, can be summarized as follows:

Scheme 4.10



Oxidations were carried out to about 10% conversion as measured by UV absorption at 234 nm. Product distributions of 2'-5' obtained from oxidation of 1P,2L-PC [18] and mixtures of diLPC [19], diP-PC [20] and cholesterol [21] are presented in Table 4.2.

Table 4.2

Product Distribution of Lecithin Autoxidation at 37°C

no.	experimental conditions	lecithins[a] oxidized	2[b]	3	4	5	$\frac{tc}{tt}$
1	emulsion[c]	18	0.21	0.28	0.20	0.31	0.69
2	neat	19	0.18	0.22	00.27	0.22	1.25
3	emulsion	19	0.30	0.24	0.26	0.20	1.26
4	emulsion	19;20(5:1)[d]	0.26	0.24	0.25	0.25	1.04
5	emulsion	19:20(4:2)	0.25	0.24	0.25	0.25	0.93
6	emulsion	19:20(3:3)	0.23	0.27	0.21	0.29	0.77
7	emulsion	19:20(2:4)	0.19	0.31	0.20	0.31	0.63
8	emulsion	19:20(1:5)	0.17	0.31	0.18	0.34	0.54
9	emulsion	19:21(8:1)	0.23	0.22	0.30	0.25	1.12
10	emulsion	19;21(8:2)	0.20	0.21	0.29	0.30	0.95
11	emulsion	19:21(8:4)	0.25	0.23	0.27	0.24	1.12
12	emulsion	19:21(8;6)	0.23	0.22	0.27	0.29	0.99
13	emulsion	19;21(8:8)	0.24	0.22	0.26	0.28	0.98

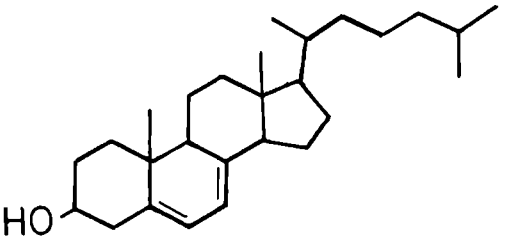
[a] 18=1P,2L-PC; 19=diL-PC; 20=diP-PC

[b] 2=13tc LA-OOH; 3=13tt LA-OOH; 4=9tc LA-OOH; 5=9tt LA-OOH

[c] Lipid concentration was 15 mM in 50 mM KCl.

[d] Ratio of 19,20 and 21 with total phospholipid concentration 15 mM.

The ratio of C-9 to C-13 substituted products was essentially 1:1 in all cases. The 13 t,c + 9 t,c/13 t,t + 9 t,t ratio, however, was substantially dependent on the nature of the medium. Addition of the completely saturated lecithin, diP-PC, to the bilayer favors t,t product formation. Similarly, addition of cholesterol to the lecithin bilayer enhances the formation of t,t products, although to a lesser extent. Provitamin D_3 (7-dehydrocholesterol) is known to be incorporated into lecithin bilayers(27), and has been reported to enhance the rate of autoxidation when mixed with linoleic acid in solution or emulsion(28).



7-dehydrocholesterol

However, unlike cholesterol it was found to strongly enhance the formation of tc hydroperoxides. Thus a tc/tt ratio as high as 3.2 was found when a mixture of diLPC and 7-dehydrocholesterol (6:4) was oxidized for 19 hr (5% diene formation) at 37°C in 50 mM KCl. These results are summarized in Table 4.3.

Table 4.3

Product Distribution of diL-PC + 7-Dehydrocholesterol (7-DHC) cooxidation at 37°C.

no.	7-DHC:diL-PC	2[a]	3	4	5	tc/tt
14	1:4	.34	.16	.35	.15	2.2
15	2:4	.37	.14	.35	.14	2.6
16	2.7:4	.35	.11	.40	.15	2.9
17	3:4	.37	.11	.39	.13	3.2

aDistribution of isomers present were based on two independent experiments; accuracy of tc/tt values is ± 0.1.

4.2.2 MICROSCOPE STUDIES OF LECITHIN EMULSIONS

In order to investigate the fate of lipid mixtures in emulsions, we studied their appearance under the microscope (Nikon, 250-1000x, polarizers, phase contrast). The data are summarized in Table 4.4 We are aware of the fact that similarity in appearance with known bilayer systems is not a guarantee that the unknown mixture has in fact a bilayer structure as well. Many other aggregation forms that appear homogeneous may very well exist. However this technique is a useful screening method in determining whether unknown mixture may form bilayer emulsions. Additional evidence from other methods such as the oxidation studies described here are, however, required. If possible, calorimetric studies (DSC) seem to be a method of choice, as well. Polyunsaturated lipids, however, tend to have

very low transition temperatures (T_t) which can make T_t measurements impossible due to limitations of the instrument. From the mixtures studied here, lecithin/linoleic acid mixtures were chosen to be investigated in greater detail by the oxidation method described next.

Table 4.4

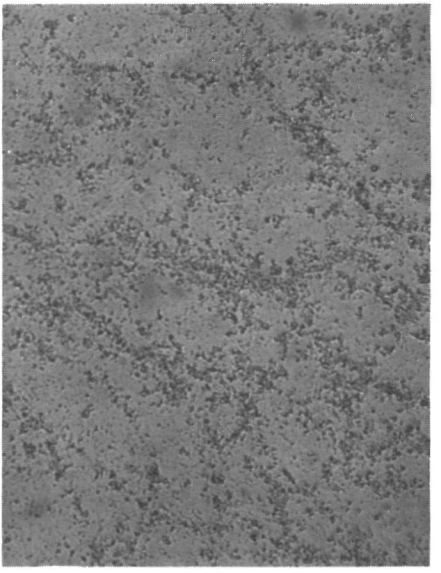
Appearance of aqueous dispersions of lipid mixtures at 30°C

lecithin	fatty acid	solvent)2	microscop-ically)3	macroscop-ically	as in photo#
egg lecithin	--	KClaq/pH7.0/A.D.	homogeneous	homogeneous	1-4
diLPC	--	id	homogeneous	homogeneous	1-4
diPPC	--	id	large crystals	heterogeneous	5
egg lecithin	AA	KClaq	large aggregates	heterogeneous	6-7
egg lecithin	AA	pH7.0	homogenecus	homogeneous	1-4
diLPC	PA	KClaq/pH7.0	homogeneous	homogeneous	1-4
diLPC	LA	id	homogeneous	homogeneous	1-4
diPPC	LA	pH7.0	homogeneous	homogeneous	1-4
diPPC	AA	KClaq/pH7.0	spherical vesicles/drops	homogeneous	8

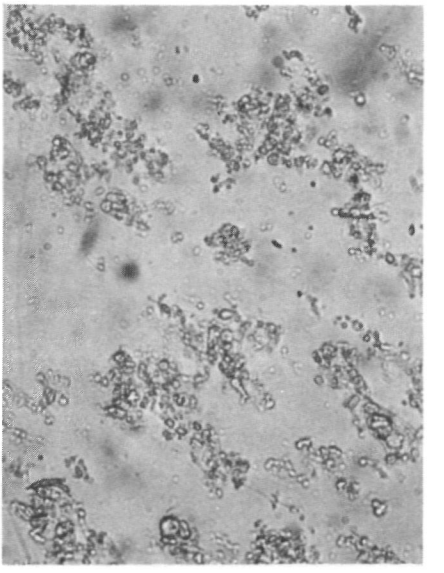
)1 in all cases lecithin/fatty acid ratio was 1:1.

)2 KClaq: 50 mM KClaq; pH7.0: 0.1 M phosphate buffer pH 7.0; A.D.: distilled water.

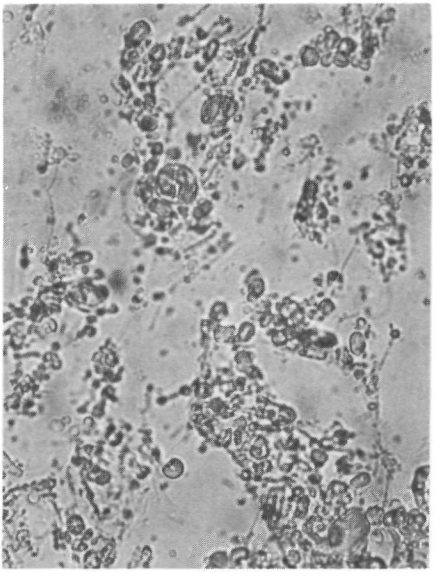
)3 25-1000 x magnification.



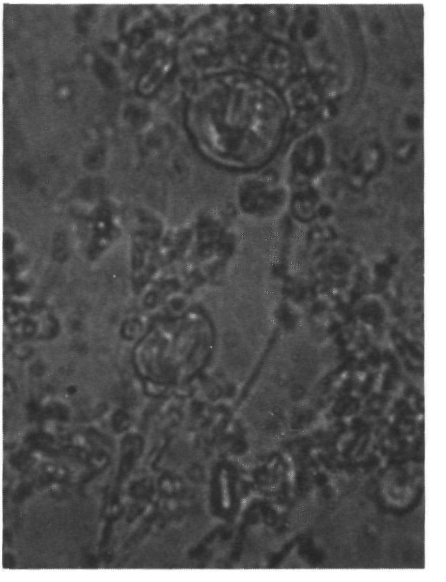
1: egg lecithin, 50 mM KCl, (130x).



2 : egg lecithin, 50 mM KCl, (260x).

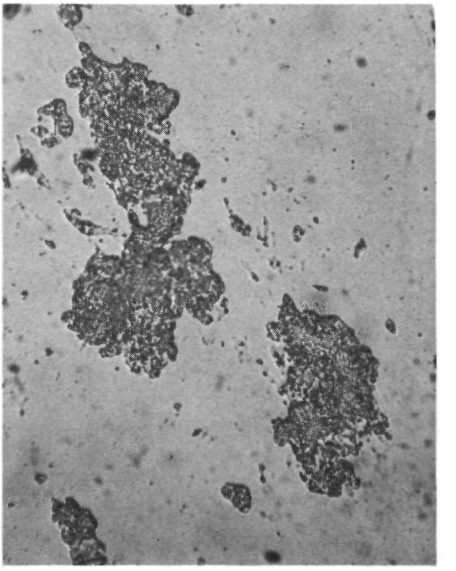


3: egg lecithin, 50 mM KCl, (520x).

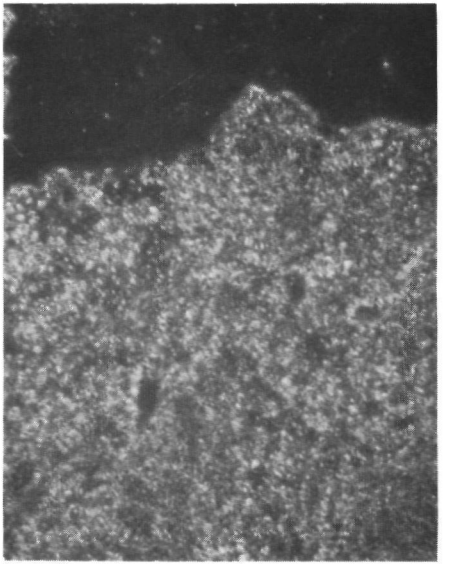


4: egg lecithin, 50 mM KCl, (1300x).

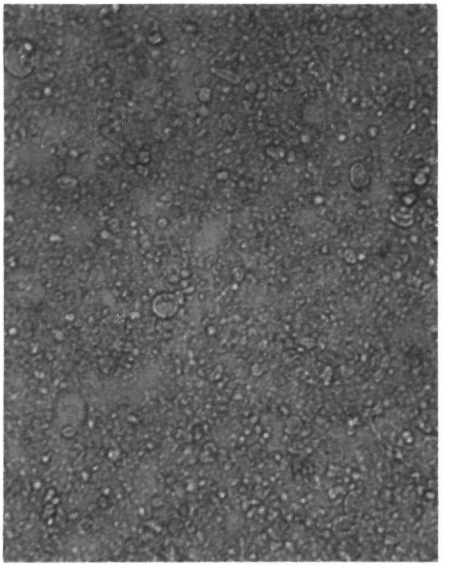
5: diP-PC, 50 mM KCl, (52x)



6: egg lec aq + AA, 50 mM KCl, (52x)



7: egg lec aq + AA, 50 mM KCl, (260x).



8: diP-PC + AA, pH 7.0 (260x)

4.2.3 COOXIDATIONS OF LINOLEIC ACID, diLPC AND diPPC

Several physical studies (DSC, NMR. diffusion, permeabilty)(29-31) indicate that fatty acids can be incorporated into phosphatidylcholine bilayers without disrupting its structure. Marbrey and Sturtevant(29) conclude from calorimetric experiments (DSC) that only fatty acids with the same chain length give optimum incorporation into the bilayer. Although we could not measure a transition temperature for diL-PC itself over a temperature range of +10° to -60°C, we did measure a transition of a diL-PC + Linoleic Acid mixture (1:1) at -27°C. This mixture did not show a melting point for linoleic acid as was observed for aqueous linoleic acid alone. Microscope studies (Nikon, 25-1000x, polarizers, phase contrast) showed no differences in this mixture when compared with aqueous diL-PC.

To further investigate lecithin/fatty acid systems we studied the cooxidation of linoleic acid and various lecithins in aqueous emulsion. Thus lecithin-linoleic acid mixtures were emulsified with a Vortex mixer and in all cases reported here a homogeneous emulsion was formed. A phosphate pH 7.0 buffer was used instead of aqueous KCl in analogy with Marbrey and Sturtevant's DSC experiments. Table 4.5 presents tc/tt ratios of both LH and diL-PC fractions of cooxidations of lecithins with linoleic acid. The fatty acids were separated from the lecithins by column chromatography (silica gel). The tc/tt ratio of the lecithins represents the 9 tc/tt ratio since the 13 tt percentage is unreliable when phospholipase A-2 is used in combination with pH 7.0 phosphate buffer (unpublished results).

Table 4.5

tc/tt Ratio of diL-PC, diP-PC and LH Cooxidations in Buffer pH 7.0.

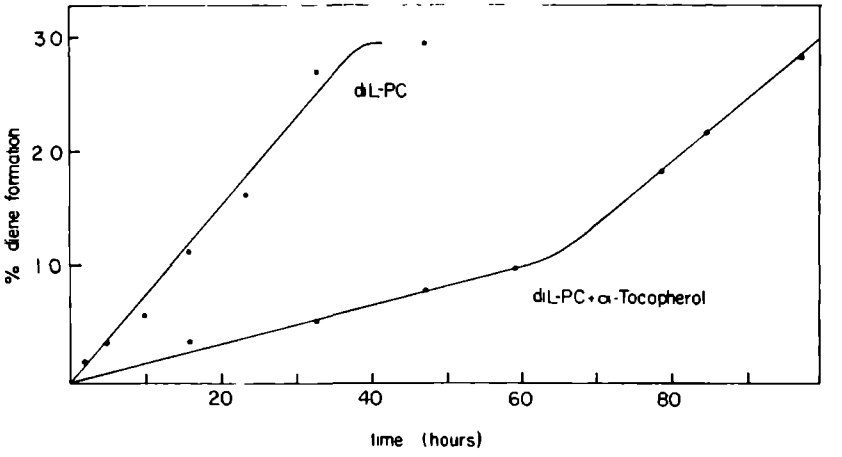
Lipids oxidized			tc/tt ratio*	
diP-PC	diL-PC	LH	diL-PC	LH
-	8	1	1.35	1.41
-	4	1	1.48	1.53
-	1	1	1.43	1.37
4	4	1	0.89	0.85
1	1	1	0.93	0.86
1	-	1	-	0.58

*accuracy of reported numbers is app 0.1, based on independently reproduced results in these and other experiments.

4.2.4 COOXIDATIONS OF DIL-PC AND α-TOCOPHEROL

DiL-PC (15 mM) was cooxidized at 37°C with α-tocopherol (5 and 10 mole %) as a homogeneous emulsion. Doubly distilled water was used instead of aqueous KCl, since the latter solvent was found to initiate autoxidation at a relatively fast rate, presumably due to impurities in the salt. The initial oxidation was much slower in doubly distilled water and the reaction was therefore easier to monitor. Microscope studies did not show any difference between aqueous KCl emulsions and the same lecithin in doubly distilled water. Autoxidation was self-initiated and diene conjugation was followed over time by UV absorption at 234 nm.

Figure 4.3 UV absorption at 234 nm as a function of time for diL-PC and diL-PC + α-tocopherol (10 mole percent) autoxidation. 20 μL 15 mM lecithin samples were diluted with 3.0 mL EtOH and their UV spectra recorded.



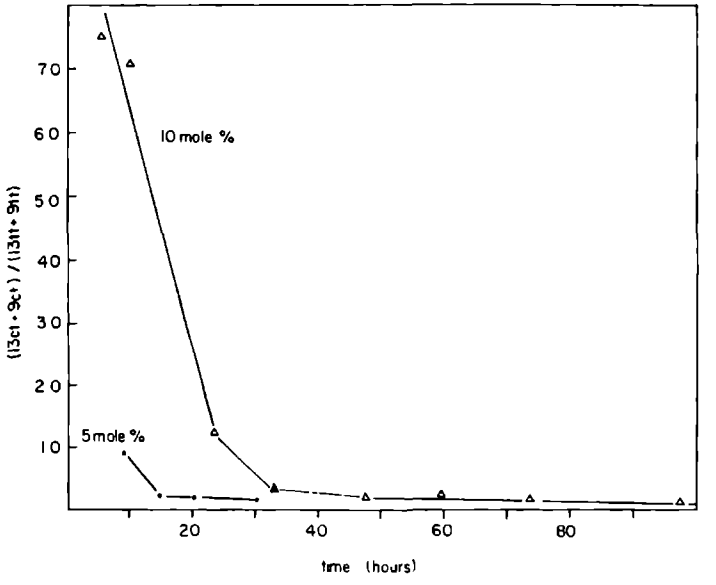
At different time intervals the lecithin hydroperoxides were converted to the corresponding hydroxy methyl linoleates, and their product distribution determined by HPLC. The unprecedented high tc/tt ratio of the products in the early stages of the cooxidation shows clearly the extremely efficient H donating ability of α-tocopherol in the bilayer (Table 4.6, Fig. 4.4).

Table 4.6

Product distribution of diL-PC + α-tocopherol (10 mole %) cooxidation

time (hours)	2	3	4	5	tc/tt
5.5	50	0.6	49	0.7	76
10	51	0.7	48	0.7	71
24	45	4	48	4	11.6
33	33	16	45	4.9	3.7
48	33	15	35	16	2.2
60	35	17	32	18	1.9
98	28	20	29	23	1.3

Figure 4.4 tc/tt ratio of hydroperoxides formed in diL-PC + α-tocopherol cooxidation vs. time.



Unlike all other lecithin oxidations, the cooxidation of α-tocopherol and diL-PC is very much dependent on the extent of oxidation. Since α-tocopherol has a phenolic H that is very easily abstracted it is oxidized much faster than diL-PC and therefore the concentration of α-tocopherol decreases dramatically with time. When all tocopherol has been consumed, the oxidation resumes its normal pattern (Fig. 4.3).

4.3 DISCUSSION

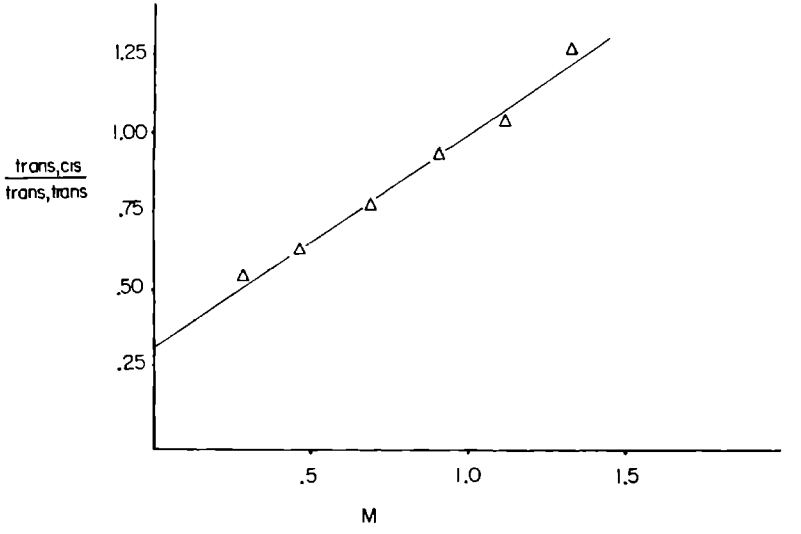
4.3.1 AUTOXIDATION OF 1P,2L-PC AND diL-PC IN THE PRESENCE OF DIP-PC, CHOLESTEROL, 7-DEHYDROCHOLESTEROL

Lecithins, neat or in aqueous emulsion, exist as bilayers of molecules with their apolar tails in the hydrophobic inner region and their polar phosphate-choline headgroup in the hydrophylic outer region of the double layer. Above their gel-liquid crystalline transition temperature they act as a two dimensional liquid, i.e., lecithin molecules diffuse laterally but are almost immobile otherwise. When mixed with water their transition temperature drops but their molecular packing stays essentially the same. Thus microscopically lecithin and water are two phases, however, macroscopically the mixture will appear homogeneous.

On a molecular level the concentration of a lecithin is therefore always the same, i.e., independent of the water content. Based on a lecithin density of 1.03 and a molecular weight of 782, we calculated the concentration of diL-PC neat or in emulsion to be 1.32 M. Similarly one can calculate the concentration of diL-PC in a mixture of diP-PC + diL-PC.

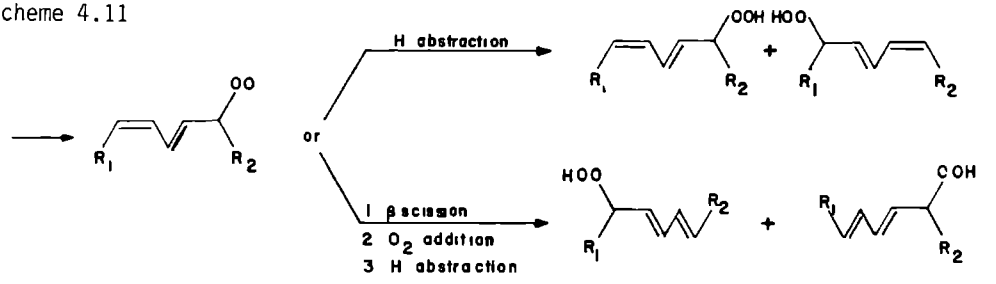
One can then plot the tc/tt ratio of PC hydroperoxides from the autoxidation of such a mixture versus the concentration of diL-PC analogous to Fig. 4.1.

Figure 4.5 Product ratio of trans,cis/trans,trans hydroxy fatty acids formed in autoxidations of diL-PC + diP-PC in emulsion as a function of diL-PC bilayer-concentration.



Autoxidation (37°C) of a 15 mM emulsion of diL-PC gives tc/tt product ratios (1.26) even greater than those obtained in the oxidation of neat linoleic acid(1,12). The highly ordered liquid crystalline state of the lecithin may facilitate intermolecular H abstraction resulting in a higher tc/tt ratio (see Scheme 4.11).

Scheme 4.11



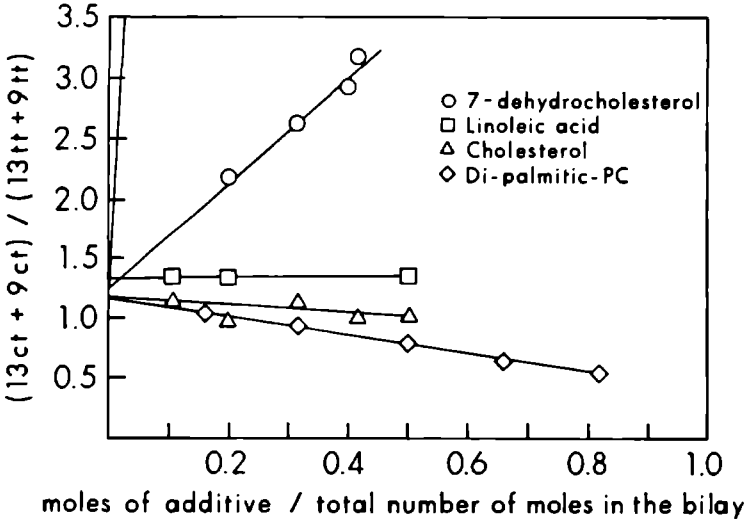
Dilution of dilinoleic lecithin with dipalmitic lecithin gives rise to a decrease in the tc/tt product ratio. Palmitate, being a saturated fatty acid, is a poor H-atom donor and the β scission pathway (leading ultimately to tt products) competes more effectively with H-atom abstraction as the concentration of linoleate decreases and that of palmitate increases. Linoleic acid has 2 bisallylic H's and is therefore a good H donor. It is evident from Fig. 4.5 that the autoxidation of diL-PC mixed with diP-PC takes place in a similar fashion as the autoxidation of linoleic acid diluted with benzene (Fig. 4.1). Thus the unimolecular β scission pathway resulting in the formation of t,t hydroperoxides competes with the bimolecular H abstraction pathway which leads directly to tc products. Simple competition kinetics lead to eq. 4.1 which thus can be applied to linoleic acid as well as linoleate containig lecithins. From Fig. 4.5 one can calculate α = 0.24 and k_p/k_β = 0.52. Since there are two linoleate residues per diL-PC molecule the effective k_p/k_β ratio per linoleate in a bilayer oxidation at 37°C is 0.26. This compares to a linoleic acid bulk phase oxidation value of 0.30. The intercept of the tc/tt vs [LH] plot leads to a value for α = 0.24 which is rather high when compared to linoleic acid bulk phase oxidation (α = 0.12). However, one has to realize that when extrapolating to [diL-PC] = 0, the [LH] at that point is far from 0, since diL-PC has two linoleic acid residues per

molecule. Thus the value for α as calculated for diL-PC may represent not only the distribution coefficient $\alpha/1-\alpha$ in eq. 4.1, but also an intramolecular H abstraction term.

It is interesting to note that a 1:1 mixture of diP-PC + diL-PC gives a product ratio (0.77) close to that observed in autoxidation of 1P,2L-PC (0.69). There thus appears to be little difference between intra and intermolecular linoleate with regard to the overall product stereochemistry observed.

Similarly cholesterol was mixed with diL-PC at various ratios. Cholesterol had a similar effect as diP-PC although less dramatic and the effect does not appear to be exactly linear (Fig. 4.6). We did not investigate this phenomena in more detail, since the effect was small compared to the accuracy of our methods.

Figure 4.6 Product ratio of t,c/t,t hydroxy fatty acids formed in cooxidations of diL-PC with diP-PC, cholesterol, 7-dehydrocholesterol and linoleic acid, in emulsion, as a function of mole percentage of the additive.



A much more dramatic effect was found when 7-dehydrocholesterol was co-oxidized with diL-PC. Trans-cis product formation was highly favored when 7-dehydrocholesterol was added to the diL-PC bilayer. A tc/tt ratio as high as 3.2 was found when 43 mole % of this steroid was present in the bilayer. We therefore conclude that 7-DHC is a much better H donor than diL-PC under the conditions described here. Assuming k_β is the same as in diL-PC + diP-PC oxidations, one can calculate k_p for 7-DHC in a bilayer autoxidation, as a function of k_p for diL-PC [$(k_p(7\text{-DHC}) \sim 5k_p(\text{diL-PC})$]. From the studies described here, we thus conclude that diL-PC bilayer autoxidation is analogous to linoleic acid bulk phase autoxidation. That is, bilayer components affecting the overall hydrogen donating ability of the medium also affect the tc/tt ratio of the hydroperoxides formed. Palmitate and cholesterol being poor H donors enhance tt product formation, while 7-DHC being a good H donor due to its 1.3 cyclohexadiene unit, gives rise to a decrease in tt products. The somewhat high tc/tt ratios for the bilayer oxidations as compared to bulk phase oxidations at high linoleate concentration may be due to a more facilitated H transfer in the highly ordered bilayer. In the low concentration range the apparent higher tc/tt ratio for lecithin oxidations may be caused by the fact that even at infinite dilution each linoleate residue is in close proximity of another linoleate residue, due to the molecular structure of diL-PC.

4.3.2 COOXIDATION OF LECITHINS AND LINOLEIC ACID

It has been reported that palmitic acid is incorporated into a diP-PC bilayer without disrupting the liquid crystalline structure(29). It was suggested that the bulkiness of the PC head group makes the

incorporation of a fatty acid with equal chain length favorable, as suggested by a higher transition temperature for the mixture as compared to the PC alone. Our results indicate that diL-PC + linoleic acid mixtures behave similarly. Thus the transition temperature diL-PC + LA (1:1) was found to be higher (-28°C) than for diL-PC alone (<-60°C). Moreover, cooxidations of mixtures of diL-PC, diP-PC and LA show a very similar product distribution as do diL-PC + diP-PC oxidations. In all cases was the tc/tt ratio for the hydroperoxides formed from incorporated linoleic acid essentially the same as the tc/tt ratio for the hydroperoxides formed from diL-PC itself. Furthermore, dilution with diP-PC has the same effect when mixed with LA, LA + diL-PC or diL-PC alone. The somewhat higher tc/tt ratios of the diL-PC + LA cooxidations could result from the closer fatty acid chain packing and therefore more efficient H atom transfer.

Interestingly, a mixture of diP-PC and LA appears to form a bilayer liquid crystalline structure, as suggested by its homogeneous appearance macroscopically and microscopically and by its oxidation product distribution; however, neither one forms a homogeneous emulsion alone, at 37°C.

4.3.3 COOXIDATION OF diL-PC WITH α TOCOPHEROL

The autoxidation of polyunsaturated fatty acids has been shown to give a different product distribution depending upon the total H donating ability of the medium. Thus, linoleic acid cooxidation resulted in tc/tt ratios varying from 0.24 to 3.5 depending on the concentration and the cosubstrate present. Similarly we have found that in bilayer cooxidations, the product distribution can be varied from predominantly tt hydroperoxide

formation (with diP-PC added) to almost exclusively tc product formation when α tocopherol is added. It has been demonstrated that α-tocopherol readily incorporates into phospholipid bilayer(33) and our data suggest it acts as an extremely good H atom donor in the bilayer. This observation is in agreement with the very high k_{inh} ($23.5 \pm .5 \times 10^5$ M^{-1} s^{-1}) found for the antioxidant effect of α-tocopherol(34). Because of the large difference between the k_p's of diL-PC and α-tocopherol, a reasonably accurate estimation of k_p for α-tocopherol cannot be made, since α-tocopherol will oxidize much faster than diL-PC, and therefore the tocopherol concentration during the reaction is unknown.

Interestingly, in the highly ordered bilayer structure where tocopherol can be expected to be located with its polar phenolic OH in the hydrophilic region of the bilayer and with its apolar tail in the hydrophobic region, there seems to be no difference between 13-OO• - α-tocopherol and 9-OO• - α-tocopherol interaction, as demonstrated by the 13 tc/tt and 9 tc/tt ratios, which are virtually the same. If peroxy radicals are polar enough to diffuse to the hydrophilic surface region of the bilayer where the active site of α-tocopherol is present, as suggested by Barclay and Ingold(24), one might expect a differerce between the 13 and 9 peroxy radical, in the rate at which they diffuse to the surface region for reasons of conformational preference and steric hindrance. None of our diL-PC + α-tocopherol cooxidations showed this effect.

In fact none of our autoxidation studies show significant regioselectivity. Both the 13tc+tt/9tc+tt and the $\frac{13tc/9tc}{13tt/9tt}$ ratios are in all cases 1.0 ± 0.1. It appears from the studies described here that in relatively slow processes such as autoxidation of PUFA's, lecithin bilayer vesicles can be considered as micro containers of neat lipids. However one

must realize that biological membranes exist as combinations of saturated and unsaturated lecithins, a variety of other phospholipids, fatty acids, cholesterol and other steroids, α-tocopherol and other tocopherol isomers and proteins of various sorts. The structural organization in such a complicated but more rigid system may very well be different, and may affect autoxidation processes as well. Studies involving multicomponent synthetic membranes as well as natural membranes are therefore called for.

4.4 EXPERIMENTAL

Fatty acids were obtained from NuChekPrep (Elysian, MN), cholesterol, 7-dehydrocholesterol and Phospholipase A-2 from Sigma, Glycerophosphoryl choline-$CdCl_2$ complex, 1-stearic-lysolecithin from Serdary Labs (Canada) and α-tocopherol from Eastman Kodak and were used without further purification. The lecithin synthesis, autoxidation procedure and analysis of the products were analogous to the procedures described in Chapter 3. Changes and additions are given below.

AUTOXIDATION

Emulsions were prepared according to the method described in Chapter 3. It was only possible to obtain homogeneous emulsions of diL-PC and diP-PC if the ratio diL-PC/diP-PC was greater than or equal to 1:5. The emulsions were stirred magnetically and kept at 37 ± 1°C with an oil bath and thermoregulator. All emulsion oxidations were stopped before diene conjugation was 10%, usually after 16-22 hr.

RP-HIGH PRESSURE LIQUID CHROMATOGRAPHY

A Waters 10 μ Bondapak column was used for reverse phase chromatography (RP-HPLC) of the lecithins. The solvent used was methanol:water 95:5. Detection was by uv absorption at 206 nm or refractive index (RI) for detecting lecithins and by uv absorption at 234 nm for more sensitive detection of the oxidized lecithins. In all cases lecithins were purified immediately before starting the oxidations. Extreme care was taken to prevent oxidation during RP-HPLC purification, by keeping the collected fractions cold (0°C) and oxygen free (Argon). DiL-PC treated this way showed no detectable peroxidation by RP-HPLC, det. uv 234 nm. All cooxidation mixtures could be analyzed without the RP-HPLC isolation of the hydroperoxide fraction, and were hydrolyzed or transmethylated after reduction of the unpurified reaction mixture.

ANALYSIS OF THE LECITHIN HYDROPEROXIDES

The product hydroperoxides were dried *in vacuo* at ambient temperature, reduced with triphenyl phosphine and hydrolyzed with Phospholipase A_2.The α-tocopherol cooxidations, however, were reduced with $NaBH_4$ in anhydrous methanol and transmethylated with KOH/MeOH(35). The two methods are essentially equivalent and only for practical reasons was one preferred over the other in certain cases. Thus all unpurified diL-PC autoxidations gave clean HPLC traces of the (methyl)linoleate hydroxides. The reduced fatty acid + lecithin co-oxidation mixtures were separated either by silica gel column chromatography or by extraction (MeOH + H_2O/hexane + ether, 1:1:1:1, v/v) to give a fatty acid fraction and a lecithin fraction which was then hydrolyzed. Both were characterized by HPLC.

HPLC CHARACTERIZATION OF THE FATTY ACID HYDROXIDES

A Whatman 5 μ Partisil analytical silica column was used for analysis of the fatty acid hydroxides. The solvent used was hexane/isopropanol, 995/5 for the hydroxy methylesters and hexane/isopropanol/acetic acid, 989/10/1 for the hydroxy fatty acids. Detection was by UV absorption at 234 nm. The product distribution was calculated based on the ε's for the individual hydroxymethyl linoleates (7). The ε's for the hydroxy linoleates were found to be essentially the same as for the methyl esters.

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CHAPTER 5

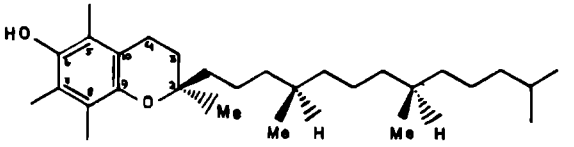
THE COOXIDATION OF 1 STEARIC, 2 ARACHIDONIC PHOSPHATIDYLCHOLINE WITH α TOCOPHEROL

5.1 INTRODUCTION

5.1.2 VITAMIN E

Self medication with megadoses of vitamin E is spreading in the USA on the basis of self-perceived benefits with unknown risks. A body of knowledge is emerging, however, which suggests that in certain situations small doses of vitamin E, for example as vitamin E rich foodsubstances, may be useful. In this introduction we will refer to several studies related to this matter.

Vitamin E is a fat soluble vitamin, also called tocopherol, of which eight naturally occurring isomers have been isolated and their structure determined. Alpha-tocopherol is usually the most important isomer isolated from natural sources and also the most active. Natural α-tocopherol has the 2R,4'R,8'R configuration(1).



(2R,4'R,8'R)α-tocopherol

A significant amount of evidence indicates that the primary function of vitamin E in vivo is that of a lipid antioxidant(2-3). Probably the most direct evidence to substantiate this theory is that lipid peroxides have been found in tissues of vitamin E deficient animals(5). It is assumed that vitamin E acts as an in vivo lipid antioxidant protecting unsaturated fatty acids in tissue lipids against peroxidation. The presence of fatty acid peroxides especially in structural lipids could result in the widespread tissue damage observed in vitamin E deficiency(6). Mary of the vitamin E deficiency symptoms seem to be related to its antioxidant activities, particularly the maintenance of membranes. Examples of such deficiency symptoms are: muscular dystrophy (rabbit), red blood cell hemolysis (man), fetal resorption (rats), mycardial degeneration (dog, rabbit), steatorrhea (man), and encephalo malacia (chick)(7-9). All the deficiency symptoms mentioned here appear to be related to an oxidative disintegration of a membrane, and suggest an antioxidant role for vitamin E.

The synergistic action of another antioxidant, vitamin C (ascorbic acid) and tocopherol also supports the antioxidant role for the tocopherols(10-11). Water soluble ascorbic acid is reported to regenerate fat soluble tocopherols after they have been oxidized. An important role for Vitamin C therefore seems to be the maintenance of vitamin E levels in tissues.

Vitamin E is suggested to protect against certain toxic drugs such as Adriamycin. The clinical use of this important cancer therapeutic agent is compromised by its potential lethal cardiotoxicity. It increases the peroxidation of cardiac lipids by initiating free radical mediated chain

reactions, which can be blocked by tocopherols(12-13).

Some investigations of the antioxidant activity of vitamin E have centered on studies of the ability of non-tocopherol antioxidants to alleviate or prevent various manifestations of vitamin E deficiency. In some cases non-tocopherol antioxidants were ineffective in replacing vitamin E; however, it is possible that insufficient amounts were given or the antioxidant replacing vitamin E was not as lipid soluble or for some other reason did not reach its site of activity. One has to realize that α-tocopherol has been shown to be an extremely efficient lipophilic chain breaking antioxidant(14), and may therefore not have been replaced with an adequate antioxidant. There has been some question as to whether the main (bio)chemical mode of action of vitamin E is related to its antioxidant activity brought about by the fact that some of the deficiency symptoms could be alleviated by dietary supplementation of selenium. It was recently discovered however that one important function of selenium is its role as an integral part of the enzyme glutathione peroxidase(15,16). Thus it now appears that vitamin E in cellular and subcellular membranes is the first line of defense against peroxidation of vital phospholipids. Even with adequate vitamin E, however, some peroxides are formed. Selenium, in glutathione peroxidase, is a second line of defense that destroys these peroxides before they have an opportunity to cause damage to the membranes(4).

In addition to its antioxidant activity, vitamin E seems to have a specific physical molecular mode of action as well. Lecithin molecules interact with α-tocopherol in monolayers of phospholipids at the air-water interface exhibiting a substantial increase in surface pressure(17-20). All of the tocopherols penetrated into monolayers more readily as

unsaturation in the phospholipids was increased. Similarly, mean molecular areas for monolayers consisting of mixtures of lecithins and tocopherols were determined. It was found that mixed monolayers of tocopherol with distearic phosphatidylcholine followed the additivity rule with respect to their mean molecular areas. In other words, these two molecular species mixed ideally in the monolayers and therefore showed no evidence of any molecular interactions. By contrast, tocopherol exhibited negative deviations in plots of mean molecular area against molar composition in mixed films with dioleic phosphatidylcholine and also in particular in mixtures with diarachidonic phosphatidylcholine. The latter finding showed that α-tocopherol and the polyunsaturated phospholipid are packed more closely together than in an ideally mixed film, indicating the existence of a molecular interaction between the two components of this monolayer that is not available to mixtures of α-tocopherol and diS-PC. In related experiments α-tocopherol was observed to decrease the permeability to glucose and to chromate ions of liposomes prepared from phospholipids containing a relatively high proportion of arachidonic acid residues.

The antioxidant activity and structural physico chemical interaction should both be considered when relating vitamin E's mode of action to deficiency symptoms. It may very well be that the combination of the two aspects of tocopherol activity makes it sufficiently different from other antioxidants that they cannot replace α-tocopherol as a vitamin. The effect described in the results of this chapter seems to suggest this point as well.

Since modern man tends to eat more polyunsaturated fatty acids which require vitamin E as an antioxidant protector, he may need to re-establish if his diet provides sufficient amounts of this vitamin. Of

even greater concern in this matter is the fact that food processing causes a significant decrease in vitamin E levels. Flour bleaching with chlorine and chlorine dioxide destroys any residual tocopherols(21), and losses of up to 90% of α-tocopherol content were found in processing of other cereal products(22). Moreover, modern man is exposed to many sources of free radicals(2,3,23) for which tocopherol can act as a detoxification agent.

A need for additional protection against increasing sources of free radical oxidation and possibly a decrease in the dietary ingestion of tocopherols suggest the need to recommend a higher vitamin E consumption. Although quantitative data to support this recommendation is lacking, recent reports seem alarming enough to make a review of one's diet recommended. One should be aware, however, that large doses of tocopherols inhibit controlled enzymatic oxidations as well (lipoxygenase)(24), which generate products (HPETE's) that have a definite physiological function (see Chapter 2). Megadoses of vitamin E therefore may not be beneficial.

5.1.2 MECHANISM OF ARACHIDONIC ACID BULK PHASE AUTOXIDATION

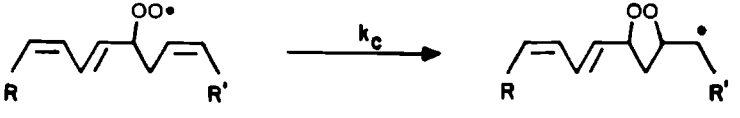
Arachidonic acid is the precursor to a variety of oxygenated metabolites, which have important physiological functions(25-38). Enzymatic pathways lead to biologically active hydroperoxides(25-29), leukotrienes(29-33), and prostaglandins(34-38) (see Fig. 1.1). Peroxy free radicals (ROO•) have been proposed as discrete intermediates in the biosynthetic conversion of arachidonic acid to the enzymatic products(35-38): nonenzymatic oxidation pathways to a variety of arachidonic acid metabolites have also been established(30-46). It is therefore of great interest both for synthetic purposes as well as to

acquire knowledge about the biosynthetic pathways, that the mechanism of arachidonic acid autoxidation has recently been studied in more detail. Based upon the information gained for linoleic acid autoxidation, an extensive study aimed at understanding arachidonic acid autoxidation pathways was carried out in our laboratory. The results of these recent studies as well as other studies concerning arachidonic acid oxidation can be summarized as follows:

1) Six major conjugated diene hydroperoxides, hydroperoxy-eicosatetraenoic acids (HPETE's), have been isolated and identified in arachidonic acid autoxidation (see Chapter 2). These major products all have trans,cis conjugated diene stereochemistry with hydroperoxide substitution being at carbons 5,8,9,11,12, and 15. Pairs of these products derive from initial abstraction of an H atom attached to the carbon allylic to both positions of the products, e.g., 11 and 15 HPETE derive from H abstraction at carbon 13 of arachidonic acid, 12 and 8 HPETE from C_{10}, and 9 and 5 from C_7 H abstraction.

2) Intramolecular addition of peroxy radicals to double bonds to form cyclic peroxides has been demonstrated to result in monocyclic(43-47) and bicyclic endoperoxides(43) as well as serial cyclization products (42,48) and epoxy alcohols(47).

Scheme 5.1



Thus radical cyclization may occur if a remote double bond is present in the peroxy radical substrate.

3) A study investigating the distributin of tc hydroperoxides formed from arachidonic acid autoxidation as a function of KP (see Chapter 4), was recently caried out in our laboraory by K. Smith(49).

Mixtures 0.1 M in arachidonic acid and 0.1 M in linoleic acid were oxidized under air or oxygen in solutions of 1,4-cyclohexadiene-benzene. Linoleic acid was added as an internal standard in the oxidation since the 9 and 13 trans,cis and trans,trans hydroperoxides of linoleic acid are the only significant products formed in bulk phase autoxidation at low conversion. Oxidation was initiated by di-t-butylperoxyoxalate and the extent of oxidation, of fatty acids and cyclohexadiene, was generally maintained at less than 2%. Fatty acid hydroperoxide products were analyzed by high performance LC on a 5 μ silica column with solvent hexane:isopropanol:acetic acid/990:10:1. Products analyzed by LC were the 5,8,9,11,12 and 15 HPETE's from arachidonic acid and the 13-trans,cis and 13-trans,trans hydroperoxides from linoleic acid, two products chosen as internal standards for the oxidation. The product distribution for HPETEs formed from arachidonic acid with reference to the two linoleic products is presented in Table 5.1. We note with interest that the 5 and 15-HPETE compounds are the major products of arachidonic acid oxidation, while HPETE's substituted at internal positions, 8,9,11 and 12 are formed to a lesser extent.

Table 5.1

Product Distribution of Arachidonic Acid Hydroperoxides Formed in Autoxidation of Mixtures of 1,4-Cyclohexadiene Benzene at 30°C

	5-HPETE[b]	8-HPETE	9-HPETE	11-HPETE[c]	12-HPETE	15-HPETE
Solvent	13tc+13tt	13tc+13tt	13tc+13tt	13tc+13tt	13tc+13tt	13tc+13tt
n[d]	24	12	22	24	24	24
97% CHD[a]	0.825	0.72	0.765	1.0	0.61	1.23
20% CHD	0.72	0.32	0.37	0.55	0.29	1.11
10% CHD	0.59	0.19	0.25	0.34	0.17	0.91
7% CHD	0.57	0.16	0.18	0.27	0.13	0.88
5% CHD	0.51	0.14	0.17	0.21	0.11	0.76

[a]CHD = 1,4-cyclohexadiene

[b]Ratio of t,c HPETE from archidonic acid vs. 13-hydroperoxides from linoleic acid, t,c and t,t

[c]A shoulder on the 11-HPETE peak was subtracted from the integrated area. This shouler was shown to be 15 t,t-HPETE

[d]Total number of analyses carried out for each HPETE isomer.

A general mechanism for triene or tetraene fatty acid autoxidation was established based upon the data presented above. Autoxidation of polyunsaturated fatty acids or esters that contain three or more double bonds introduces an additional competitive process, peroxy radical cyclization, that must be considered in the mechanistic scheme. This modified format, shown in Scheme 5.1, suggests that three independent

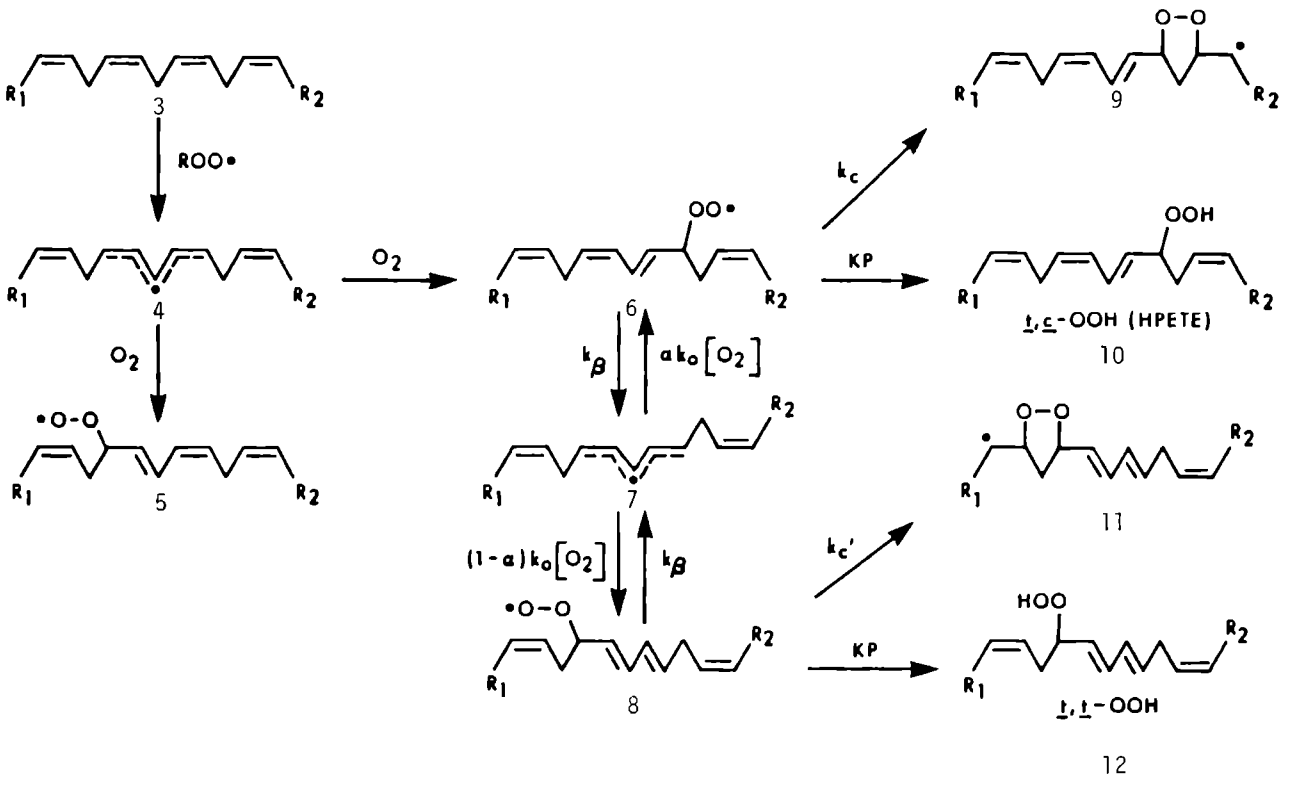
pathways control product distribution in triene or tetraene fatty acid oxidation. Two of these processes, β scission and cyclization, are unimolecular events while the third, hydrogen atom transfer, is critically dependent on KP of the medium of oxidation. Scheme 5.2 is thus essentially identical to Scheme 4.7 with the exception that the trans,cis peroxy radical 6 may undergo cyclization with rate k_c and the trans,trans peroxy radical 8 may cyclize with rate k_c'. Steady-state kinetics on 7 and 8 leads to $[8]/[6] = (1-\alpha)k_\beta/(\alpha k_\beta + KP + k_c')$. One should also note that only products are considered here that are derived from the peroxy radical 6 and assume that a similar, but independent expression could be written for 5.

Products analyzed in the study of arachidonic acid oxidation described in this report (Table 5.1) are the trans,cis hydroperoxide (HPETE) compounds. An expression for HPETE vs. all products was derived from peroxy radical 6 as a function of constants outlined in Scheme 5.2. In Eq. 5.1 $HPETE_o$ is inserted for "all products from 6".

$$\frac{\text{All products from 6}}{\text{HPETE}} = \frac{[6]\,KP + [6]\,k_c + [8]\,k_c' + [8]\,KP}{[6]\,KP}$$

or

$$\frac{HPETE_o}{HPETE} = 1 + \frac{k_c}{KP} + \frac{(1-\alpha)k_\beta\, k_c'}{(\alpha k_\beta + k_c' + KP)KP} + \frac{(1-\alpha)k_\beta}{(\alpha k_\beta + k_c' + KP)} \qquad \text{(eq. 5.1)}$$


R1
R2
3
ROO•
4
O2
5
•O-O
6
OO•
7
8
kβ
α ko [O2]
(1 - α) ko [O2]
kc
kc'
KP
9
O-O
10
OOH
t,c-OOH (HPETE)
11
12
HOO
t,t-OOH

Scheme 5.2

Consider the following specific cases where Eq. 5.1 might be applied:

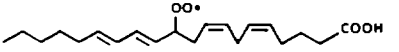
1) KP = ∞, 1/KP = 0; with this boundary condition HPETE = $HPETE_o$. Thus at very high KP only t,c hydroperoxides are formed, and product distribution should only be a function of the relative rates of H-abstraction from the 3 bisallylic C's.

2) $k_c = k_c' = 0$; in this case the mechanism outlined in Scheme 5.2 reduces to Scheme 4.7, a mechanism used to describe diene fatty acid oxidation where no radical cyclization is possible. Equation 5.1 yields Equation 5.2 in this special case and Eq. 5.2 ($HPETE_o$ = t,c + t,t) is equivalent to Eq. 4.1.

$$\frac{HPETE_o}{HPETE} = 1 + \frac{(1-\alpha)k_\beta}{\alpha k_\beta + KP} \qquad \text{(eq. 5.2)}$$

3) $k_c = 0$, $k_c' \neq 0$; this condition is met for the trans, cis peroxy radical precursor to 15-HPETE, 13 or the corresponding radical intermediate leading to 5-HPETE. In both of these radicals no remote double bond is readily available for cyclization from the trans,cis radical ($k_c = 0$) while the

13

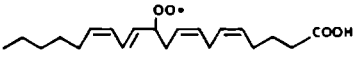


14

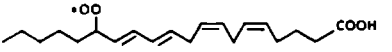
associated trans,trans radical, 14, may undergo cyclization ($k_c' = 0$). If, additionally, $k_c' + KP >> \alpha k_\beta$ (a condition that is generally met in oxidations using cyclohexadiene co-substrate) then Eq. 5.1 reduces to Eq. 5.3.

$$\frac{HPETE_0}{HPETE} = 1 + \frac{(1-\alpha)k_\beta}{KP} \qquad \text{(eq. 5.3)}$$

4) $k_c' = 0$, $k_c \neq 0$; this condition is met for the trans,cis peroxy radical pecursors to 11-HPETE, 15, and 9-HPETE. The corresponding trans,trans peroxy radicals, of which 16 is an example, derived from 15, do not have a cyclization pathway



15



16

available. If we again assume that $KP >> \alpha k_\beta$ then Eq. 5.1 is simplified to Eq. 5.4

$$\frac{HPETE_o}{HPETE} = 1 + \frac{k_c}{KP} + \frac{(1-\alpha)k_\beta}{KP} \qquad \text{(eq. 5.4)}$$

5) $k_c' \neq 0$, $k_c \neq 0$; this final condition is met for peroxy radical precursors to 12-HPETE and 8-HPETE. Assuming KP + k_c' >> αk_β then Eq. 5.4 may again be used to describe this specific situation.

The general equation describing the mechanism outlined in Scheme 5.2, Eq. 5.1, thus provides specific expressions (Eqs. 5.2, 5.3, and 5.4) that may be used in considering product distributions obtained in the autoxidation of diene, triene, and tetraene fatty acids. Each particular trans,cis peroxy radical and its coupled β scission peroxy radical (e.g. 13 and 14) must be analyzed with regard to the potential for radical cyclization and by such considerationthe choice of an appropriate equation is made.

5) Based upon the kinetic parameters derived from the arachidonic acid autoxidation product distribution study as outlied under 4-5, product distribution simulation plots were obtained as shown in Fig. 5.1 and 5.2. The figures represent the product distribution as a function of KP for a cyclizable HPETE radical such as 12-HPETE and one for a non-cyclizable HPETE such as 15-HPETE.

Fig. 5.1 Product distribution simulation plot for 12-HPETE radical.

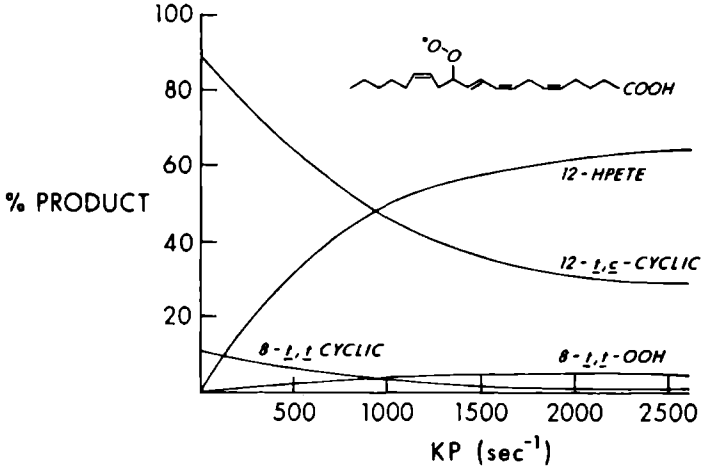
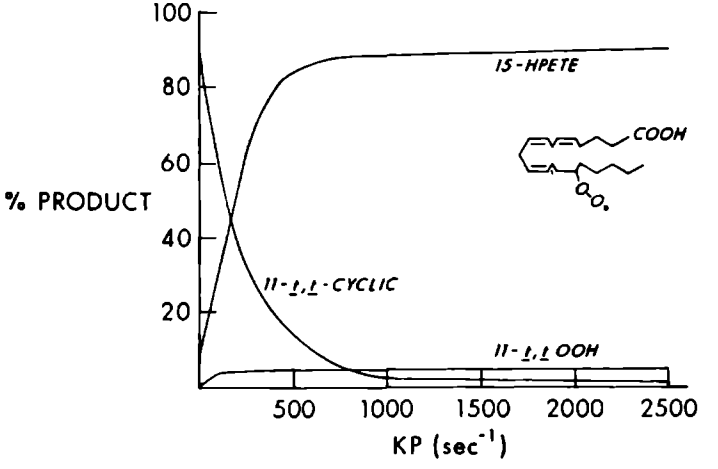


Fig. 5.2 Product distribution simulation plot for 15-HPETE radical.

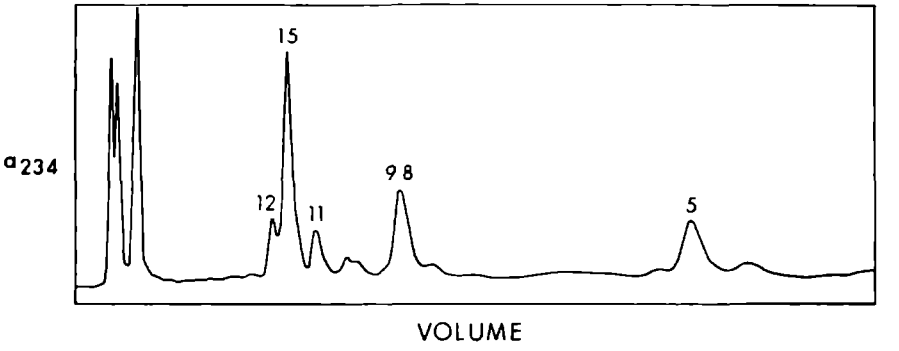


5.2 RESULTS(50)

5.2.1 1S,2A-PC AUTOXIDATION

1 Stearic, 2 Arachidonic Phosphatidyl Choline (1S,2A-PC, 15 mM) was autoxidized at 37°C as a homogeneous emulsion in aqueous KCl (50 mM). Autoxidation was self initiated and stopped after five hr (5% diene formation, by UV absorbance at 234 nm). Shown in Fig. 5.3 is the HPLC trace of the methyl hydroxy-arachidonates (Me-HETE's) obtained from 1S,2A-PC autoxidation.

Fig. 5.3 HETE methyl esters frm 1S,2A-PC emulsion autoxidation



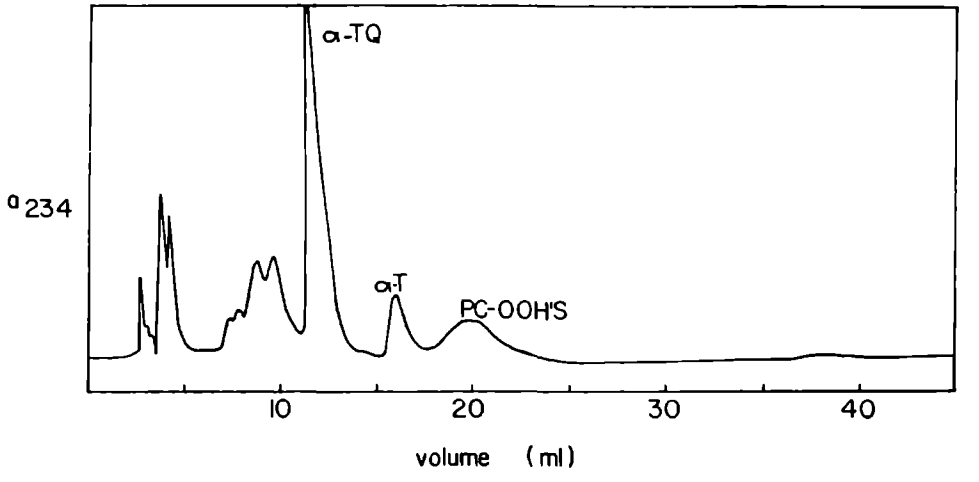
From the different workup methods described in Chapter 3, $NaBH_4$ reduction followed by transmethylation with KOH/MeOH was found to give the cleanest HETE analysis by HPLC (Fig. 5.3). Unfortunately the 9 and 8 substituted isomers are not readily separated as the methyl esters. The position of hydroxy substitution on the twenty carbon chain is indicated in the figure and based on comparison with Me-HETE traces obtained from methyl

arachidonate autoxidation (see Chapter 2). Thus 12, 15, 11, 9, 8, and 5 HPETE-lecithins were formed from the autoxidation of 1S,2A-PC emulsion. The product distribution of the hydroperoxides found is given in Table 5.2 (see Discussion) and was based on three independent reactions.

5.2.2 COOXIDATION OF 1S,2A-PC WITH α TOCOPHEROL

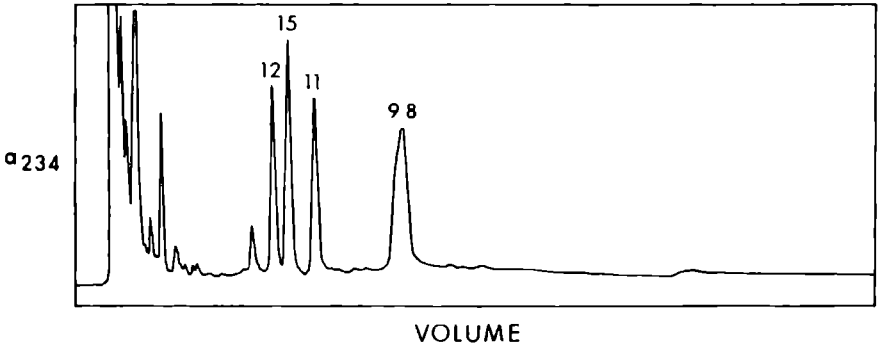
1S,2A-PC (15 mM) was cooxidized at 37°C with α-tocopherol (25 mole %) as a homogeneous emulsion in doubly distilled water(50). Autoxidation was self initiated and the reaction followed by RP-HPLC (Fig. 5.4). Under the conditions of the chromatography (95% MeOH, 2 mL/min), 1S,2A-PC elutes at 38 mL, the oxidized PC fraction at 19 mL, α-tocopherol at 16 mL and α-tocopherolquinone at 12mL.

Figure 5.4 RP-HPLC of 1S,2A-PC + α-tocopherol cooxidation in aqueous emulsion



When α-tocopherol was almost completely consumed (app. five hr), all fractions were analyzed by uv and the fraction with a uv absorption maximum at 234 nm collected, reduced and transmethylated. The HPLC trace of the product mixture obtained this way is shown in Fig. 5.5.

Figure 5.5 HETE methyl esters frm 1S,2A-PC + α-tocopherol cooxidation in aqueous emulsion



One would expect all the isomers to be present in about equal amounts, due to the extremely high H donating ability (k_{inh},k_p) of α-tocopherol, as reported in this dissertation for the cooxidation of diL-PC and α-tocopherol (see Chapter 4). Fig. 5.6 shows that this is essentially the case for the 12,15,11,9, and 8 isomer, however the 5-HETE which corresponds to the 5 hydroperoxy lecithin is surprisingly absent. In three independent cooxidations the same distribution was found at the initial stage of the oxidation. Upon checking our methods we have not been able to find an error in methodology. Looking for a possible explanation, we worked up a crude cooxidation mixture directly before RP-HPLC

purification. The normal phase chromatogram obtained in this way again showed the absence of the 5 isomer. We also found that the 5-O-lactone and the 5 keto derivative, both of which we independently synthesized, had not been formed.

The largest peak in Figure 5.4 was found to be α-tocopherol-quinone. It appears to be the main α-tocopherol oxidation product, based upon refractive index detection. It should be emphasized that the lack of the formation of the 5-HPETE was found only at the initial stages of autoxidation. After most α-tocopherol was consumed the 5-HPETE was observed to go back to normal levels in a matter of hours. Furthermore the effect was observed at lower levels of α-tocopherol (10 mole %) as well, however, the isolation and characterization of the HPETE's was more difficult and therefore unpractical since less product was formed.

5.3 DISCUSSION

It was concluded from the arachidonic acid + cyclohexadiene cooxidation studies, as summarized in the introduction of this chapter, that the H atom donating ability of the medium (KP) is an important factor affecting product distribution. The non cyclizable 15 and 5 isomers are the main HPETE's formed at low KP, while at high KP the differences in the amounts of HPETE's formed are much smaller (see Table 5.2). One has to be aware of this phenomena when comparing autoxidations in bilayer and bulk phase. Table 5.2 allows us to compare 1S,2A-PC emulsion oxidation with a bulk phase oxidation at equivalent KP. It is evident from the table that 1S,2A-PC bilayer autoxidation is analogous to arachidonic acid autoxidation in 7% CHD. The product distributions exhibit a similar pattern, with only small differences that do not appear significant.

Table 5.2

Product Distribution of HETE Methyl Esters Obtained from Archidonate Autoxidations)[1]

	KP)[2] (s^{-1})	5 (%)	8+9 (%)	11 (%)	12 (%)	15 (%)
Arachidonic acid)[3] (7% CHD, benzene)	241	24	16	12	6	40
Arachidonic acid)[3] (97% CHD, benzene)	3336	16	29	19	12	24
1S,2A-PC)[4], aqueous	233)[5]	24	22	9	8	37
1S,2A-PC + a-toc (25 mole%))[4], aqueous	-	-	35	21	19	26

)1 It was assumed that the e's for all isomers are the same.

)2 Based on values calculated by K. Smith, et al.(49).

)3 at 30°C, reported by K. Smith, et al.(49).

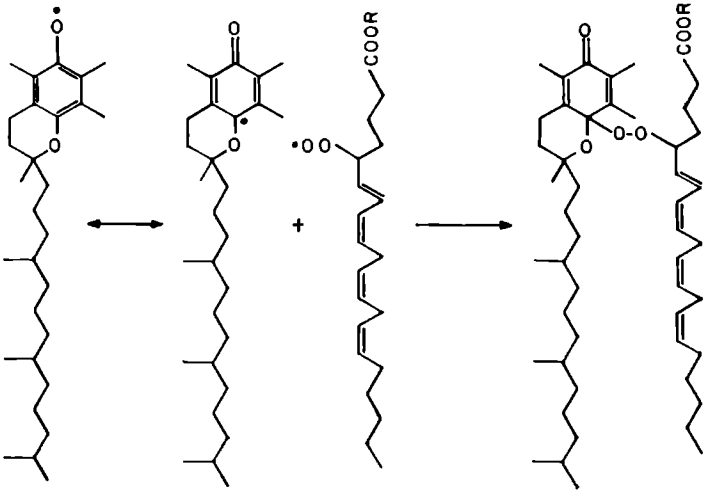
)4 at 37°C

)5 based on KP for arachidonic acid

If the 1S,2A-PC emulsion oxidation is carried out with added α-tocopherol, a dramatic change in product distribution is observed from that noted in oxidations with no added tocopherol. First, as shown in Table 5.2, the 12,15,11,9, and 8 HPETE products are formed in almost equal amounts. This pattern fits the notion that α-tocopherol acts as a good H atom donor in the bilayer and traps the 8,9,11, and 12 peroxy radicals

before cyclization can occur. A second change in product distribution of 1S,2A-PC emulsion oxidation with added α-tocopherol is the virtual absence of 5-HPETE products. We have looked for a trivial explanation for this observation but have found none. For example, we reasoned that perhaps 5-O-lactone is somehow generated during oxidation or workup. This was not the case, however, as judged by HPLC comparison of the product mixture with an authentic sample. Furthermore, appropriate control experiments suggest that the lack of 5 substituted products in the α-tocopherol experiments was not due to its loss in the workup (see Experimental).

Scheme 5.2



17 18 19 20

One explanation for the loss of 5-substituted arachidonic acid products in bilayer oxidations with added α-tocopherol is the possibility of selective trapping of arachidonyl peroxy radicals by the tocopherol

phenoxy radical. The α-tocopherol radical 17 formed by H atom donation would be expected to reside in the bilayer with the polar phenoxy oxygen near the hydrophilic head-groups of the phospholipid. We suggest that the 5-substituted peroxy radical 19 might be preferentially scavenged by 17 due to proximity of the 5 peroxy radical (closest to the head group of any of the radicals generated) and 17. Products substituted at position 5 would thus disappear from the system by preferential scavenging of the peroxy radical, presumably at the position para to phenoxy oxygen, 18. We figured that somehow we may have missed 20 when analyzing the HPLC fractions by UV. It seemed reasonable to assume that 20 upon transmethylation with KOH/MeOH would decompose to 5 keto-6,8,11,14-eicosatetraenoate and α-tocopherol quinone. We therefore subjected a crude 1S,2A-PC + α-tocopherol cooxidation mixture to our usual transmethylation procedure (KOH/MeOH), and subsequently analyzed it by HPLC. However, we did not detect any 5 keto-6,8,11,14-eicosatetraenoate, when comparing HPLC retention volumes of the crude transmethylated reaction mixture with independently synthesized 5 keto-6,8,11,14-eicosatetraenoate.Nevertheless addition of an α-tocopherol radical to preferentially the 5 position of 1S,2A-PC seems to be the most likely explanation for the lack of 5-HPETE formation. Positionally selective 5 peroxy-arachidonate-α-tocopherol interaction can be expected based upon phospholipid-α-tocoherol molecular interactions, as proposed by Lucy, et. al (see Introduction of this Chapter). The authors noted that the physical α-tocopherol-lecithin interaction was particularly evident when high proportions of arachidonic acid were incorporated into the phospholipids(17-20), as is the case for the 1S,2A-PC + α-tocopherol experiments described in this chapter. The authors suggest that the physical interaction between α-tocopherol and arachidonic acid may anchor

vitamin E in the membranes.

It is puzzling, however, what the fate of the suggested adduct 20 would be under the conditions of the workup procedure. Systematic analysis of all products formed in the cooxidation of an arachidonic acid containing lecithin and α-tocopherol is therefore called for.

It should be noted that the high proportion of α-tocopherol present in the experiments described here (10-25 mole %) is not necessarily beyond physiological levels. Tocopherol concentrations as high as 10 mole % are present in some membranes, such as the retinal rod outer segments which are highly unsaturated(51).

The lack of 5-substituted products with added α-tocopherol is of biological interest. An important class of compounds, the leukotrienes, are derived from 5-HPETE and it has recently been reported that lecithins containing 5-hydroxy substituted arachidonate esters are generated enzymatically. The specific inhibition of formation of 5-HPETE products by α-tocopherol in these model membrane oxidations studied here may thus have biological significance, and may represent a unique physiological role for α-tocopherol.

5.4 EXPERIMENTAL

The experimental part of the 1S,2A-PC autoxidation is very similar to Chapter 4. Changes and additions are given below:

The cooxidation of 1S,2A-PC and α-tocopherol was followed by RP-HPLC over a period of time and when less than 20% of the original α-tocopherol was left unoxidized, samples were taken and the RP-HPLC fraction with a UV maximum at 234 nm was collected and transmethylated to

determine product ratio. Appropriate control experiments assured us that this RP-HPLC purification did not cause the loss of the 5 substituted product in α-tocopherol cooxidations. RP-HPLC of 1S,2A-PC autoxidations carried out without added α-tocopherol led to normal product distribution thus indicating that 5 HPETE products were not lost in the workup of the α-tocopherol reactions.

TOCOPHEROL QUINONE

The fraction containing α-tocopherolquinone (Fig. 3) was characterized as follows: It was found to be peroxide negative, it had a faint yellow appearance as well as a uv absorbance maximum at 262 (shoulder) and 268 nm(52). ^{1}H NMR spectroscopy was performed on a Bruker 250 MHz (Δ, $CHCl_3$): 3.33 (s,broad), 2.55 m, 2.04 (s), 2.00 (s), 1.0-1.8 (complex mixture of multiplets), 0.86 (t).

SYNTHESIS OF 5 KETO-6,8,11,14 EICOSATETRAENOIC METHYL ESTER

Methylarachidonate was exposed to air for about 48 hr. The mixture of hydroperoxides formed was subjected to HPLC purification and the 5-HPETE isolated. Five to ten milligram 5-HPETE was reacted with one mL pyridine and two drops acetyl chloride at 0°C for 15 min. One mL water was added and the mixture extracted with 50 mL ether/hexane (1:1). The organic layer was washed with 4% HCl aq. and dried with $MgSO_4$. UV(EtOH or Hexane): λ_{max} 273 nm.

SYNTHESIS OF 5-O-5,8,11,14 EICOSATETRAENOIC LACTONE

The 5-O-5,8,11,14 eicosatetraenoic lactone was obtained from arachidonic acid via the iodolactone(53-55). Thus arachidonic acid was

reacted with 8 aq I_2, 15 aq KJ, and 5 aq $KHCO_3$ in THF/H_2O (1:1) for four to six hr. One has to be careful to use unoxidized arachidonic acid of great purity (99+%). The reaction mixture was worked up by adding a thiosulfate solution to reduce excess iodine and subsequent extraction with ether. The ether layer was washed with $KHCO_3$ and H_2O and dried with Na_2SO_4. The iodolactone was purified by Florisil column chromatography.

^{1}H nmr (Δ,$CDCl_3$, 80 Mhz) 5.1-5.6 (m,6H,vinyl), 3.8-4.2 (2H,CHJ and -CHOC(=O)-), 2.6-2.9 (m,4H,bisallylic), 2.3-2.6 (m,2H,-CH_2-C(=O)-), 1.7-2.1 (m,6H), 1.1-1.4(m), 0.7-1.0 (m). The Iodolactone was then reacted with 2.5 eq 1.8-Diazobicyclo 5.4.0 undec-7-ene (DBU) in benzene. The reaction mixture was magnetically stirred for about 12 hr under argon. The lactone was isolated by silica column chromatography.

λ_{max} 236 nm, ^{1}H nmr (Δ,$CDCl_3$, 80 MHz) 5.3-6.7 (m,6H,vinyl), 4.9 (m,1H,C(=O)-O-CH), 2.7-3.1 (m,4H,bisallylic), 2.3-2.7 (m,2H,O-C(=O)-CH_2), 1.7-2.2 (m), 1.2-1.4 (m), 0.7-1.0 (m).

The HPLC retention volume of the lactone was distinctly different from the methyl HETE's; it was eluted between the 9, 8, and 5 methyl HETE's.

5.5 REFERENCES

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SUMMARY:

The air oxidation of linoleic acid (18:2), arachidonic acid (20:4), and their methyl esters was investigated. A mixture of hydroperoxides was obtained from the oxidation, and subsequent separation by high pressure liquid chromatography (HPLC) led to pure hydroperoxides which were analyzed by uv, ir, and GC-MS. The HPLC separation could be applied to separate all isomers from a mixture of linoleic acid and arachidonic acid hydroxides generated by autoxidation and subsequent reduction. The procedure outlined in Chapter 2 can be used to prepare individual hydroperoxides. The separation of the primary products of autoxidation, the fatty acid hydroperoxides, will provide a new approach in autoxidation studies, since factors affecting the product distribution in the autoxidation process can now be studied. This important aspect had, until now, not been investigated in a systematic way, and the analytical method described here affords a relatively simple way to further elucidate the mechanism of autoxidation.

An important class of polyunsaturated fatty esters are the phospholipids, such as lecithins. The potential physiological role of lecithin oxidation products is discussed in the introduction of chapter 3, and a mechanism for trans membrane transport of hexose sugars and electrolytes is proposed.

Chapter 3 further describes studies involving lecithin autoxidation. Two unsymmetrical polyunsaturated lecithins and a natural lecithin mixture from egg yolk were allowed to air oxidize and the primary products of autoxidation were isolated and characterized. 1-Palmitic-2-linoleic-phosphatidylcholine undergoes significant oxidation after 16 hr at

room temperature under air. A new phospholipid product may be isolated by reverse phase high pressure liquid chromatograhy and this HPLC fraction is shown to be made up of lipid hydroperoxides formed by free radical oxidation of the homoconjugated diene of the linoleate component of the lecithin. 1-Stearic-2-arachidonic-phosphatidylcholine undergoes a similar oxidation with the arachidonate polyunsaturated functionality being oxidized. Egg lecithin oxidizes much slower and gave rise to only linoleic acid hydroperoxides. The structure of the oxidation products was established by reduction of the hydroperoxides with triphenylphosphine, snake venom hydrolysis of the C-2 ester, and HPLC analysis of the resulting hydroxy fatty acids or their methyl esters. A RP-TLC separation of lecithin species was developed. This method proved to be useful in monitoring lecithin oxidations as well, since RP-TLC unlike NP-TLC completely separated oxidized products from the unoxidized lecithin.

The autoxidation of diL-PC in aqueous emulsion with several lipophilic cosubstrates was investigated and is described in Chapter 4. DiP-PC, cholesterol, 7-dehydrocholesterol, linoleic acid and α-tocopherol were cosubstrates in the autoxidation of diL-PC. The distribution of the products, tc and tt diene hydroperoxides, was determined and evaluated. The ratio of trans,cis/trans,trans products formed during the initial stages of the oxidation (<10%) of diLPC + diPPC, was dependent on the concentration of diLPC in the bilayer, i.e., diLPC/(diLPC + diPPC). The kinetic scheme presented for linoleic acid oxidation was consistent with the data obtained for diLPC oxidation. Thus substrates or cosubstrates with a high H donating ability (k_p) enhance t,c product formation, while in a medium with low H donating ability (k_p) tt products are formed. It was concluded that the autoxidation of polyunsaturated lecithins is very

similar to bulk phase autoxidation of polyunsaturated fatty acids. No regioselectivity in the studies described here was detected.

An important polyunsaturated fatty acid present in animals is arachidonic acid, precursor to a variety of pharmacologically active oxidation products. The autoxidation of a naturally frequently occurring arachidonic lecithin, 1S,2A-PC, was therefore investigated in aqueous emulsion. The distribution of the products, hydroperoxy eicosatetraenoic acid isomers (HPETE's), was very similar to arachidonic acid neat autoxidation. The cooxidation of 1S,2APC with α-tocopherol in aqueous emulsion, however, was significantly different from comparable neat autoxidations. The 5-HPETE was surprisingly absent in the very early stages of autoxidation, the 12,15,11,9, and 8 isomers being present in almost equal amounts. The results are discussed with respect to the mechanism of autoxidation and possible biological implications.

SUPPLEMENT: ABBREVIATIONS

PL	Phospholipids
PC	Phosphatidyl Choline, Lecithin
TG	Triglycerides
PUFA	Polyunsaturated Fatty Acid
P	Palmitic acid (16:0)
L	Linoleic acid (18:2, ω6)
S	Stearic acid (18:0)
A	Arachidonic acid (20:4, ω6)
HPETE	Hydroperoxy eicosatetraenoic acid (ester)
HETE	Hydroxy eicosatetraenoic acid (ester)
HPLA	Hydroperoxy linoleate
FA	Fatty acid
FA-OOH	Fatty acid Hydroperoxide
PC-OOH	Phosphatidylcholine Hydroperoxide
GPC-$CdCl_2$	Glycerophosphoryl Choline-Cadmium Chloride Complex
BHT	t-Butylated Hydroxy Toluene
DMAP	Dimethylaminopyridine
nmr	nuclear magnetic resonance
RI	refractive index
UV	ultraviolet
tlc	thin layer chromatography
rp-tlc	reverse phase tlc
HPLC	High Performance (Pressure) Liquid Chromatography
rp-HPLC	reverse phase HPLC
DSC	differential scanning calorimetry
k_p	rate of H abstraction

SAMENVATTING:

De basisstructuur van biologische membranen is een dubbellaag van fosfolipiden, waarin zich eiwitten bevinden. Die eiwitten hebben evenals de fosfolipiden zelf, een zekere mate van beweeglijkheid in het vlak van de fosfolipide dubbellaag, die afhankelijk is van de mate van onverzadigdheid van de in de fosfolipiden ingebouwde vetzuren. De onverzadigde vetzuren hebben evenwel ook de eigenschap gemakkelijk met zuurstof te reageren, hetgeen tot biologisch aktieve stoffen kan leiden met soms destruktieve gevolgen. De niet enzymatische reaktie van onverzadigde lipiden met zuurstof, ook wel autooxydatie genoemd, is dan ook gesuggereerd als oorzaak van velerlei pathologische processen. Een zeer geschikt model voor de bestudering van membraan autooxydatie vormen liposomen, kleine deeltjes (25-2500 nm in diameter) bestaande uit fosfolipide dubbellagen, die in essentie dezelfde structuur bezitten als cellulaire membranen. Liposomen worden gevormd wanneer fosfolipiden boven hun overgangstemperatuur (T_t) met water worden vermengd. Het hier beschreven onderzoek is er op gericht de oxydatie van membraanmodellen te bestuderen. Door structuur opheldering en kwantitatieve analyse van de produkten van de autooxydatie reaktie is getracht dit doel te bereiken. Er zijn daarom de volgende onderzoekingen gedaan:

De aan de lucht spontaan verlopende oxydatie van de meervoudig onverzadigde vetzuren linolzuur en arachidonzuur evenals hun methyl esters werd bestudeerd. Hierbij werden mengsels van hydroperoxiden verkregen die werden gescheiden door middel van hoge druk vloeistof chromatografie (HPLC), en geanalyseerd met behulp van ultra violet en infra rood spectroscopie, en massa spectrometrie. Alle isomeren van een mengsel

van linolzuur en arachidonzuur hydroxiden, verkregen door autooxydatie en reduktie konden door middel van HPLC worden gescheiden. De in hoofdstuk 2 beschreven methoden kunnen worden toegepast om individuele hydroperoxiden te synthetiseren en mengsels te analyseren.

Deze onverzadigde vetzuren werden vervolgens ingebouwd in fosfatidylcholine (PC), een veel voorkomend fosfolipide in biologische membranen. 1-Palmitoyl,2-lineoyl-PC en 1-stearine,2-arachidon-PC werden gedurende enige uren (resp. 16 en 5 uur) aan de lucht blootgesteld, waarna de autooxydatie produkten werden geisoleerd en geanalyseerd. De hydroperoxyden konden met 'reverse phase' HPLC (rp-HPLC) of rp-TLC worden gescheiden van de uitgangsstof, maar de individuele hydroperoxiden konden slechts worden geanalyseerd na reduktie en hydrolyse tot de vetzuurhydroxiden. Op dezelfde manier werd de autooxydatie van een natuurlijk mengsel van PC's bestudeerd, n.l. de in eidooier voorkomende PC's. Hoewel ei lecithine zowel linolzuur als arachidonzuur bevat, werden na autooxydatie alleen linolzuurhydroperoxiden gevonden.

De autooxydatie van dilineoyl-PC(diL-PC) liposomen en de invloed daarop van andere dubbellaagcomponenten, n.l. dipalmitoyl-PC (diP-PC), cholesterol, provitamine D_3, linolzuur en vitamine E, wordt uitvoerig beschreven in hoofdstuk 4. Bij de autooxydatie van een mengsel van diL-PC en diP-PC bleek de verhouding van de trans,cis en trans,trans produkten evenredig te zijn met de concentratie van diL-PC in de dubbellaag, d.w.z. met (diL-PC)/(diL-PC+diP-PC). Het kinetische schema voor linolzuuroxydatie bleek redelijk overeen te komen met dat voor diL-PC oxydatie. Dus substraten of cosubstraten die gemakkelijk H atomen doneren (hoge k_p) geven voornamelijk trans,cis dieen produkten, terwijl in een medium dat slechts moeilijk H atomen doneert

(lage k_p), trans,trans dieen produkten in grotere mate worden gevormd. Dit leidt tot de conclusie dat de oxydatie van meervoudig onverzadigde lecithinen in liposomen analoog is aan de autooxydatie van de vetzuren waaruit die PC's bestaan. In de hier beschreven studies werd dan ook geen regioselektiviteit gevonden.

Een belangrijk meervoudig onverzadigd vetzuur in dieren is arachidonzuur, dat verscheidene biologisch aktieve oxydatie produkten kan vormen. De autooxydatie van 1S,2A-PC werd daarom onderzocht in een liposomale oplossing. De verhouding van de hierbij gevormde hydroperoxiden was weer dezelfde als bij de autooxydatie van het vrije arachidonzuur. Tenslotte werd de cooxidatie van 1S,2A-PC met tocopherol (vitamine E) in liposomale oplossing bestudeerd. Hierbij werd wel een opvallend verschil met de autooxydatie van vrije vetzuren gevonden. Het 5-arachidonzuur-hydroperoxide bleek in de eerste fase van de autooxydatie totaal afwezig, terwijl de 12,15,11,9 en 8 isomeren in bijna gelijke mate gevormd werden. De resultaten worden verder besproken met betrekking tot het autooxydatie mechanisme en mogelijke biologische consequenties.

CURRICULUM VITAE

De auteur van dit proefschrift bezocht van 1965-1971 het Hertog Jan College te Valkenswaard (gymnasium β). In 1971 werd aan de chemie studie begonnen aan de universiteit van Nijmegen, in 1974 werd het kandidaatsexamen behaald (S2), en in 1978 het doctoraalexamen (hoofdvakken: organische chemie onder leiding van Prof Dr B. Zwanenburg en farmacologie onder leiding van Prof Dr J.M. van Rossum). Van november 1976 tot september 1977 werd het tweede gedeelte van het hoofdvak organische chemie voltooid aan de universiteit van Bowling Green, Ohio, U.S.A., bij prof Dr D.C. Neckers.

De aantekening onderwijsbevoegdheid werd behaald in 1978, na het doorlopen van de cursus didactiek onder leiding van Drs C.H. van der Ent en Drs F.J.C.M. Arnold. De aanstelling als onderzoeksmedewerker aan Duke University (Durham, North Carolina, U.S.A.) van 1 oktober 1978 tot 1 november 1981, resulteerde in het hier beschreven onderzoek onder leiding van Prof Dr N.A. Porter en Prof Dr B. Zwanenburg. Sinds 1 december 1981 is hij als wetenschappelijk medewerker verbonden aan het Antoni van Leeuwenhoekhuis (Nederland Kankerinstituut).

I

De aanbevolen dagelijkse consumptie van Vitamine E en C moet vanwege de onrustbarend toenemende blootstelling aan oxiderende milieuvervuiling worden herzien.

II

Het door Cillard et al gevonden prooxydatieve effect van hoge concentraties Vitamine E mag niet worden gezien als een eigenschap die ook op biologische systemen van toepassing is.

J. Cillard, P. Cillard, JAOCS 1980, 39-42.

III

Bij de bestudering van PC autooxydatie houden Barclay en Ingold ten onrechte geen rekening met het feit dat fosfatidyl cholines in organische oplosmiddelen micellen vormen.

L.R.C. Barclay, K.U. Ingold, J. Am. Chem. Soc. 1980, 102, 7792.
P.H. Elworthy, D.S. McIntosh , J. Pharmacy Pharmacol. 13, 663 (1961).

IV

Arnett en Gold gaan bij de analyse van de door hen gevonden chirale discriminatie in gemengde monolagen van DiP-PC en N-(α-methylbenzyl)stearamide, voorbij aan de voor de hand liggende mogelijkheid van clustervorming.

E.M. Arnett, J.M. Gold, J.A.C.S. 1982, 104, 636-639.

Bij gebruikmaking van chemotherapie moet het doseringstijdstip optimaal gekozen worden ten opzichte van de circadische variaties van de patient.

F. Halberg et al, Experientia, Vol. 29, Fasc 8, p. 909-1044 (1973).

VI

Bij onderzoek naar therapieën die gebaseerd zijn op immunologische reacties dient te worden nagegaan of manipulatie van de lipide-vloeibaarheid van de celmembranen het beoogde effect kan vergroten.

Y. Skormick, E. Danciger, R.R. Rozin, M. Shinitzky, Cancer Immunol. Immunother. (1981) 11: 93-96.

VII

Vanwege het belang van een selectieve verdeling verdienen membraan drug interacties nader te worden beschouwd als een essentieel onderdeel van rationeel drug design.

E. Goormaghtigh, P. Chatelain, J. Caspers, J.M. Ruysschaert, Biochemical Pharmacology, Vol. 29, p. 3003-3010.

VIII

Het succes van het invoeren van een middenschool is afhankelijk van de mogelijkheid tot brede individuele differentiatie en is derhalve in de huidige politieke situatie ongewenst.

IX

Ieder portret is een zelfportret.

Rob Schemkes, januari 1982.

X

De plantenwereld verdient meer respect: niet alleen is zij in staat de mens van alle mogelijke voedingsstoffen te voorzien, maar ook heeft zij de beschikking over simpele doch zeer selectieve chemische synthetische methoden die de organisch chemicus met Weenmoed doen vervullen.

Hugo Weenen

Nijmegen, 13 mei 1982.